IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:		§		
		§		
	Gila MAOR	§		
		§		
Serial No.:	10/627,739	§		
		§		
Filed:	July 28, 2003	§	Group Art Unit:	1651
		§		
For:	CULTURED CARTILAGE/ BONE CELLS/TISSUE, METHODS OF GENERATING SAME AND USES THEREOF	§		
		§		
		§		
		§		
		§		
		§	Attorney Docket:	**26243**
		§		
Examiner:	Lora Elizabeth Barnhart	§		

DECLARATION OF GILA MAOR UNDER 37 CFR 1.132

I am presently employed as researcher at the Technion-Israel Insititute of Technology, Department of Medicine, where I am an associate senior lecturer. . I received my Ph.D degree from the Technion in developmental biology,

My research focuses on Hormonal regulation of chondrogenesis. Since the beginning of my career, I have published 57 scientific articles in highly regarded journals and books, and have presented my achievements at many international scientific conferences.

I am a member of ICRS (International cartilage repair society) and Endocrinological Society.

I am a co-inventor of the subject matter claimed in the above-referenced U.S. patent application.

I have read the Official actions issued with respect to the above-identified application.

In this Official action, the Examiner has rejected claims 1-9, 11, 14, 17-23 and 108 under 35 U.S.C. § 112 first paragraph because the specification, as-filed, includes working examples in which chondrocyte culturing should be effected for at least 7 days.

The Appendix section enclosed herewith illustrates that re-differentiation of chondrocytes to collagen II+/collagen I- expressing cells already occurs after 3 days in culture, albeit to a lesser extend. These results conclusively show that the methodology described and claimed in the instant application can be utilized to make and use the claimed culturing method, thereby proving that the rejections of claims 1-9, 11, 14, 17-23 and 108 under 35 U.S.C. § 112 first paragraph are unfounded.

In re Application of: Gila MAOR
Serial No.: 10/627,739
Filed: July 28, 2003
Office Action Mailing Date: August 13, 2008

Examiner: Lora Elizabeth Barnhart
Group Art Unit: 1651
Attorney Docket: 26243

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United states Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

December 11, 2008

Gila Maor

Dr. Gila Maor
Dept. Medicine
Technion, Israel

Enc.:
CV of Gila Maor and Appendix
26243

TECHNION - FACULTY OF MEDICINE

RESUME **Date:** February /2008

1. PERSONAL DETAILS

Full Name	Gila Maor
Identification number:	0-0308477-9
Date and place of birth:	April 25, 1949, Israel
Phone no:	04-8713554; 052-8714578

2. ACADEMIC DEGREES

Degree	Year	Institute	Subject
B.Sc.	1971	Bar-Ilan University	Microbiology and biochemistry
M.Sc.	1973	Technion Faculty of Biology	Regulation of Transcription in Bacteriophages.
D.Sc.	1982	Technion Faculty of Medicine	Endocrinological regulation of skeletal growth.
Postdoc.	1988	Bar-Ilan University Faculty of Life Sciences	The involvement of interferon in skeletogenesis.

3. ACADEMIC APPOINTMENTS

From-to:	Institute	Appointment
2001-present	Technion Faculty of Medicine	Associate senior lecturer

4. PROFESSIONAL EXPERIENCE

From-to:	Institute	Area of specialization
1974-present	Technion Faculty of Medicine	Hormonal Regulation of skeletal tissue development. Morphological studies, localization of gene expression (protein and mRNA), biochemical and molecular analyses.
1971-1973	Technion- Faculty of Biology	Regulation of transcription mechanism in bacteriophage

5. RESEARCH INTERESTS

The interests of my research group concerns the endocrinilogical regulation of skeletal growth and development.

Specifically we explore the following issues:

a) The direct regulatory effects of growth hormone, IGF-1, and insulin on skeletal growth process.

b) The role played by IGF receptors and GLUT4 in mediating the IDDM-related growth retardation: its effects on gene expression.

c) The effects of acidosis (chronic renal failure) on the endochondral ossification process.

d) The role played by gonadal steroids (androgens and estrogens) in regulation of skeletal growth. Specifically we are interested in the effects of estraduol in accelarated osteogenesis and enhanced apoptosis in the growth centers of both genders.

e) Establishment a reliable chondrocytic tissue culture mimicking the normal endochondral ossification process.

6. TEACHING EXPERIENCE

1. The Cell Structure and function	No. 274107
2. Anatomy II –Limbs	No. 274118
3. Nursing - Cell Biology	No. 290.1016.1א
4. Occupational Therapy-Anatomy Limbs	No.3201105
5. Histology	No. 276200

7. MEMBERSHIP IN PROFESSIONAL SOCIETIES

National: 1. Israel Endocrine Society (IES)

2. Israel Diabetes Association (IDA)

International: 1. European Society of Pediatric Endocrinology (ESPE)

2. The Endocrine Society

3. International Bone and Mineral Society (IBMS)

4. International cartilage repair society (ICRS)

8. HONORS

Henning Andersen Prize (1988) European Society for Pediatric Endocrinology

9. STUDENTS AND INSTRUCTION

Student	Degree	Title	Year of graduate	Name of additional supervisor	Public ation No
Ruth Satran-Goldberg	M.Sc.	The effects of metabolic acidosis on skeletl responsiveness to hormones.	2002	Prof. J Green	47,
Miriam Rochberger-Ben Eliezer	M.Sc	The effects of leptin on skel4etal growth.	2002	Prof. R. Coleman	48, 43
Aviad Keren	M.Sc.	Influence of Estrogen on Endochondral Ossification.	2003	Prof. R. Coleman	
Ella Reshef	M.Sc.	The Signal transduction pathway involved in the effects of CMA on the development of skeletal tissue.	2003	Prof. R. Coleman	54
Gaya Vasiliver	M.S.c.	IGF-I Receptor Regulates GLUT4 Gene Expression and Activity in Skeletal Growth Centers	2003	Prof. E Karnieli	55
Ruth Goldberg	D.Sc.	Gene Therapy of Rheumatoid Arthritis: Local administration of Osteoprotegerin	2005	Prof. N Karin	50, 51, 54
Roni Bril	M.Sc.	Effects of IGFI receptor on the regulation of GLUT4 activity-cellular aspects .	2007	Prof. E Karnieli	
Marina Bendarski	M.Sc.	Effects of membrane and cytoplasmic estrogen receptors on skeletal growth: involvement of signal transduction in the process	2007	Prof. M Phillip	
Hila Kreizman	M.Sc.	Interaction between fibrilast growth Factor (FGF) and osteoprotegerin (OPG) in regulation of skeletal growth	---------	Prof. avner Yayon	

Student	Degree	Title	Year of graduate	Name of additional supervisor	Publication No
Shira Ben-Zvi	M.Sc.	Developing a 'Cartilage Patch' for treating articular cartilage damages in the Joints	candidate		M.Sc.

10. RESEARCH GRANTS

Research topic	Funding agency	Total grant	Grant partnership
Regulation of IGF-I activity in murine skeletogenesis (PI)	Israeli Ministry of Health Office	NIS 160,000	____
The role played by androgens in regulating maturation and apoptosis of the growth plate chondrocyte. (PI)	Israeli Ministry of Health Office	NIS 100,000 2003-2004	____
Effect of leptin on children's growth. (CI)	Israel Science Foundation	NIS 220,000 2003-2006	Prof M Phillip, Schneider's children hospital

11. SIGNIFICANT PROFESSIONAL PROJECTS

a. 1995-1998, 2006-2007: Developing anti-tumor material from reptile serum.

This project was managed by Prof. O. Binah, Prof. A Ciechanover and myself.

b. 1999-present: Developing a novel model for spontaneously differentiating chondrocyte cells, for basic science research and clinical applications.

The above development is protected by two filed patents. Based on this technology a Carticure Ltd.- a biotech start-up company was established in 2005 and is working in the premises of NGT biotechnology incubator funded by the chief scientist's budget . I serve as the entrepreneur and scientific consultant of the company.

12. PUBLICATIONS

12 a. Refereed papers

1. **Maor, G.**, and Shalitin, C. Competence of membrane-bound T4rII DNA for in vitro "late" mRNA transcription. Virology, *62:* 500-11, 1974.

2. Silbermann, M., and **Maor, G.** Mechanisms of glucocorticoid-induced growth retardation: impairment of cartilage mineralization. Acta Anat (Basel), *101:* 140-9, 1978.

3. Silbermann, M., and **Maor, G.** Effect of glucocorticoid hormone on the content and synthesis of nucleic acids in cartilage of growing mice. Growth, *43:* 273-87, 1979.

4. Silbermann, M., and **Maor, G.** Mandibular growth retardation in corticosteroid-treated juvenile mice. Anat Rec, *194:* 355-67, 1979.

5. **Maor, G.,** and Silbermann, M. In vitro effects of glucocorticoid hormones on the synthesis of DNA in cartilage of neonatal mice. FEBS Lett, *129:* 256-60, 1981.

6. Silbermann, M., Weiss, A., **Maor, G.**, and Lewinson, D. Involvement of glucocorticoids in cartilage growth and metabolism. Adv Exp Med Biol, *171:* 257-67, 1984.

7. Silbermann, M., and **Maor, G.** Receptor-mediated glucocorticoid inhibition of cell proliferation in mouse growth cartilage in vitro. Acta Endocrinol (Copenh), *108:* 343-50, 1985.

8. Kraiem, Z., **Maor, G.,** and Silbermann, M. Dexamethasone and 8-bromo-cyclic AMP depress the incorporation of [3H]thymidine into mouse condylar cartilage by different pathways. J Endocrinol, *109:* 209-13, 1986.

9. **Maor, G.**, and Silbermann, M. Supraphysiological concentrations of dexamethasone induce elevation of calcium uptake and depression of [3H]-thymidine incorporation into DNA in cartilage in vitro. Calcif Tissue Int, *39:* 284-90, 1986.

10. **Maor, G.**, and Silbermann, M. Studies on hormonal regulation of the growth of the craniofacial skeleton: IV. Specific binding sites for glucocorticoids in condylar cartilage and their involvement in the biological effects of glucocorticoids on cartilage cell growth. J Craniofac Genet Dev Biol, *6:* 189-202, 1986.

11. **Maor, G.**, von der Mark, K., Reddi, H., Heinegard, D., Franzen, A., and Silbermann, M. Acceleration of cartilage and bone differentiation on collagenous substrata. Coll Relat Res, *7:* 351-70, 1987.

12. Silbermann, M., von der Mark, K., **Maor, G.,** and van Menxel, M. Dexamethasone impairs growth and collagen synthesis in condylar cartilage in vitro. Bone Miner, *2:* 87-106, 1987.

13. Laufer, D., Ben-Shachar, D., Livne, E., **Maor, G.**, and Silbermann, M. Enhancing effects of fluoride-containing ceramic implants on bone formation in the dog femur. J Craniomaxillofac Surg, *16:* 40-5, 1988.

14. **Maor, G.**, Hochberg, Z., von der Mark, K., Heinegard, D., and Silbermann, M. Human growth hormone enhances chondrogenesis and osteogenesis in a tissue culture system of chondroprogenitor cells. Endocrinology, *125:* 1239-45, 1989.

15. **Maor, G.**, Hochberg, Z., and Silbermann, M. Growth hormone stimulates the growth of mouse neonatal condylar cartilage in vitro. Acta Endocrinol (Copenh), *120:* 526-32, 1989.

16. **Maor, G.**, Salzberg, S., and Silbermann, M. The activity of 2,5-oligoadenylate synthetase, an interferon-induced enzyme, is coupled to the differentiation state of mouse condylar cartilage. Differentiation, *44:* 18-24, 1990.

17. Hochberg, Z., Hertz, P., **Maor, G.**, Oiknine, J., and Aviram, M. Growth hormone and insulin-like growth factor-I increase macrophage uptake and degradation of low density lipoprotein. Endocrinology, *131:* 430-5, 1992.

18. Weinstock, M., Matlina, E., **Maor, G.** I., Rosen, H., and McEwen, B. S. Prenatal stress selectively alters the reactivity of the hypothalamic-pituitary adrenal system in the female rat. Brain Res, *595:* 195-200, 1992.

19. **Maor, G.**, Laron, Z., Eshet, R., and Silbermann, M. The early postnatal development of the murine mandibular condyle is regulated by endogenous insulin-like growth factor-I. J Endocrinol, *137:* 21-6, 1993.

20. **Maor, G.**, Silbermann, M., von der Mark, K., Heingard, D., and Laron, Z. Insulin enhances the growth of cartilage in organ and tissue cultures of mouse neonatal mandibular condyle. Calcif Tissue Int, *52:* 291-9, 1993.

21. *Maor, G.*, Hochberg, Z., and Silbermann, M. Insulin-like growth factor I accelerates proliferation and differentiation of cartilage progenitor cells in cultures of neonatal mandibular condyles. Acta Endocrinol (Copenh), *128:* 56-64, 1993.

22. Yadid, G., **Maor, G.,** Youdim, M. B., Silberman, M., and Zinder, O. Autoradiographic localization of strychnine-sensitive glycine receptor in bovine adrenal medulla. Neurochem Res, *18:* 1051-5, 1993.

23. Felzen, B., Shilkrut, M., Less, H., Sarapov, I., **Maor, G.**, Coleman, R., Robinson, R. B., Berke, G., and Binah, O. Fas (CD95/Apo-1)-mediated damage to ventricular myocytes induced by cytotoxic T lymphocytes from perforin-deficient mice: a major role for inositol 1,4,5-trisphosphate. Circ Res, *82:* 438-50, 1998.

24. Rihani-Bisharat, S., **Maor, G.**, and Lewinson, D. In vivo anabolic effects of parathyroid hormone (PTH) 28-48 and N-terminal fragments of PTH and PTH-related protein on neonatal mouse bones. Endocrinology, *139:* 974-81, 1998.

25. Sprecher, E., Bergman, R., Sprecher, H., **Maor, G.**, Reiter, I., Krivoy, N., Drori, S., Assaraf, Y. G., and Friedman-Birnbaum, R. Reduced folate carrier (RFC-1) gene expression in normal and psoriatic skin. Arch Dermatol Res, *290:* 656-60, 1998.

26. Sprecher, E., Bergman, R., Sprecher, H., **Maor, G.**, Reiter, I., Krivoy, N., Drori, S., Assaraf, Y. G., and Friedman-Birnbaum, R. The reduced folate carrier (RFC-1) gene is expressed in the murine epidermis. Arch Dermatol Res, *290:* 394-6, 1998.

27. Youssef, S., Wildbaum, G., **Maor, G.**, Lanir, N., Gour-Lavie, A., Grabie, N., and Karin, N. Long-lasting protective immunity to experimental autoimmune encephalomyelitis following vaccination with naked DNA encoding C-C chemokines. J Immunol, *161:* 3870-9, 1998.

28. **Maor, G.,** Segev, Y., and Phillip, M. Testosterone stimulates insulin-like growth factor-I and insulin-like growth factor-I-receptor gene expression in the mandibular condyle--a model of endochondral ossification. Endocrinology, *140:* 1901-10, 1999.

29. **Maor, G.**, and Karnieli, E. The insulin-sensitive glucose transporter (GLUT4) is involved in early bone growth in control and diabetic mice, but is regulated through the insulin-like growth factor I receptor. Endocrinology, *140:* 1841-51, 1999.

30. Sprecher, E., Bergman, R., Meilick, A., Kerner, H., Manov, L., Reiter, I., Shafer, Y., **Maor, G.**, and Friedman-Birnbaum, R. Apoptosis, Fas and Fas-ligand expression in melanocytic tumors. J Cutan Pathol, *26:* 72-7, 1999.

31. Green, J., and **Maor, G**. Effect of metabolic acidosis on the growth hormone/IGF-I endocrine axis in skeletal growth centers. Kidney Int, *57:* 2258-67, 2000.

32. Wildbaum, G., Westermann, J., **Maor, G.**, and Karin, N. A targeted DNA vaccine encoding fas ligand defines its dual role in the regulation of experimental autoimmune encephalomyelitis. J Clin Invest, *106:* 671-9, 2000.

33. Youssef, S., **Maor, G.**, Wildbaum, G., Grabie, N., Gour-Lavie, A., and Karin, N. C-C chemokine-encoding DNA vaccines enhance breakdown of tolerance to their gene products and treat ongoing adjuvant arthritis. J Clin Invest, *106:* 361-71, 2000.

34. Assady, S., **Maor, G.**, Amit, M., Itskovitz-Eldor, J., Skorecki, K. L., and Tzukerman, M. Insulin production by human embryonic stem cells. Diabetes, *50:* 1691-7, 2001.

35. Grunblatt, E., Mandel, S., **Maor, G.**, and Youdim, M. B. Gene expression analysis in N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mice model of Parkinson's disease using cDNA microarray: effect of R-apomorphine. J Neurochem, *78:* 1-12, 2001.

36. Grunblatt, E., Mandel, S., **Maor, G.**, and Youdim, M. B. Effects of R- and S-apomorphine on MPTP-induced nigro-striatal dopamine neuronal loss. J Neurochem, *77:* 146-56, 2001.

37. Levites, Y., Weinreb, O., **Maor, G.**, Youdim, M. B., and Mandel, S. Green tea polyphenol (-)-epigallocatechin-3-gallate prevents N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopaminergic neurodegeneration. J Neurochem, *78:* 1073-82, 2001.

38. Lewinson, D., **Maor, G.**, Rozen, N., Rabinovich, I., Stahl, S., and Rachmiel, A. Expression of vascular antigens by bone cells during bone regeneration in a membranous bone distraction system. Histochem Cell Biol, *116:* 381-8, 2001.

39. Phillip, M., **Maor, G.**, Assa, S., Silbergeld, A., and Segev, Y. Testosterone stimulates growth of tibial epiphyseal growth plate and insulin-like growth factor-1 receptor abundance in hypophysectomized and castrated rats. Endocrine, *16:* 1-6, 2001.

40. Armoni, M., Quon, M. J., **Maor, G.**, Avigad, S., Shapiro, D. N., Harel, C., Esposito, D., Goshen, Y., Yaniv, I., and Karnieli, E. PAX3/forkhead homolog in rhabdomyosarcoma

oncoprotein activates glucose transporter 4 gene expression in vivo and in vitro. J Clin Endocrinol Metab, *87:* 5312-24, 2002.

41. Levites, Y., Youdim, M. B., **Maor, G.**, and Mandel, S. Attenuation of 6-hydroxydopamine (6-OHDA)-induced nuclear factor-kappaB (NF-kappaB) activation and cell death by tea extracts in neuronal cultures. Biochem Pharmacol, *63:* 21-9, 2002.

42. Mandel, S., Grunblatt, E., **Maor, G.**, and Youdim, M. B. Early and late gene changes in MPTP mice model of Parkinson's disease employing cDNA microarray. Neurochem Res, *27:* 1231-43, 2002.

43. **Maor, G.**, Rochwerger, M., Segev, Y., and Phillip, M. Leptin acts as a growth factor on the chondrocytes of skeletal growth centers. J Bone Miner Res, *17:* 1034-43, 2002.

44. Reiter, I., Tzukerman, M., and **Maor, G.** Spontaneous differentiating primary chondrocytic tissue culture: a model for endochondral ossification. Bone, *31:* 333-9, 2002.

45. Salomon, I., Netzer, N., Wildbaum, G., Schif-Zuck, S., **Maor, G.**, and Karin, N. Targeting the function of IFN-gamma-inducible protein 10 suppresses ongoing adjuvant arthritis. J Immunol, *169:* 2685-93, 2002.

46. Youdim, M. B., Grunblatt, E., Levites, Y., **Maor, G.**, and Mandel, S. Early and late molecular events in neurodegeneration and neuroprotection in Parkinson's disease MPTP model as assessed by cDNA microarray; the role of iron. Neurotox Res, *4:* 679-689, 2002.

47. Green, J., Goldberg, R., and **Maor, G.** PTH ameliorates acidosis-induced adverse effects in skeletal growth centers: the PTH-IGF-I axis. Kidney Int, *63:* 487-500, 2003.

48. Eshet, R., **Maor, G.**, Ben Ari, T., Ben Eliezer, M., Gat-Yablonski, G., and Phillip, M. The aromatase inhibitor letrozole increases epiphyseal growth plate height and tibial length in peripubertal male mice. J Endocrinol, *182:* 165-72, 2004.

49. Gat-Yablonski, G., Ben-Ari, T., Shtaif, B., Potievsky, O., Moran, O., Eshet, R., **Maor, G.**, Segev, Y., and Phillip, M. Leptin reverses the inhibitory effect of caloric restriction on longitudinal growth. Endocrinology, *145:* 343-50, 2004.

50. Goldberg, R., Zohar, Y., Wildbaum, G., Geron, Y., **Maor, G.**, and Karin, N. Suppression of ongoing experimental autoimmune encephalomyelitis by neutralizing the function of the p28 subunit of IL-27. J Immunol, *173:* 6465-71, 2004.

51. Goldberg, R., Wildbaum, G., Zohar, Y., **Maor, G.**, and Karin, N. Suppression of ongoing adjuvant-induced arthritis by neutralizing the function of the p28 subunit of IL-27. J Immunol, *173:* 1171-8, 2004.

52. Mandel, S., **Maor, G.**, and Youdim, M. B. Iron and alpha-Synuclein in the Substantia Nigra of MPTP-Treated Mice: Effect of Neuroprotective Drugs R-Apomorphine and Green Tea Polyphenol (-)-Epigallocatechin-3-Gallate. J Mol Neurosci, *24:* 401-16, 2004.

53. Topaz O. Bergman R. Mandel U. **Maor G.** Goldberg R. Richard G. Sprecher E. Absence of intraepidermal glycosyltransferase ppGalNac-T3 expression in familial tumoral calcinosis. American Journal of Dermatopathology. *27(3):*211-5, 2005

54. Goldberg R , Ella Reshef-Bankai, Coleman R, Green J, **Maor G:** Chronic Acidosis-Induced Growth Retardation Is Mediated by Proton-Induced Expression of Gs Protein. J Bone and Mineral Research, *21(5):* 703-13, 2006.

55. Vasiliver G, Karnieli E, **Maor G**: IGF-1 Receptor Dependent Glucose transport and GLUT4 translocation in Bone. Endocrinology (submitted for publication) 2007.

56. Goldberg R, Karin N, **Maor G**: The Direct Impact of Ostroprotegerin (OPG) on cartilage development – a novel cartilage growth factor (submitted for publication) 2008

12b. Reviews

1. Silbermann M., Maor G. (1984) Organ and tissue culture of cartilage and bone. In: Methods of Calcified Tissues Preparation. G. R. Dickson, ed., Elsevier/North Holland Biomedical Press, Amsterdam, pp 467-530. Chapter in Book.

2. Invited by Prof. Z Laron to write a review article on the "Mandibular condylyle as an appropriate model to study long bone growth" for J of Pediatric Endocrinology & Metabolism.

12c. Patents

Year	Title	Status of patent
Priority Date: July,19,2001	Anti-Tumor Activity from Reptile Serum	PCT/IL : 02/000590 WO 03/007874
2003	Insulin producing cells derived from human embryonic stem cells.	Application No.143155
2003	Cultured cartilage cells/tissues, method of generating same and uses thereof.	Application No. 10/627,739

13. CONFERENCES

13a. Invited talk

1. The osteogenic differentiation of the mandibular condyle.

2. The 2nd European conference of Cartilage and Bone Remodeling. 1994, Giens, France.

13b. Contriuted talks and abstracts (Last five years)

Conference Title	Location	Date	Name of the speaker
1. Int. workshop on Calcified Tissues	Eilat	Febr. 1999	Maor G
2. ESPE	Brussels	Sep. 2000	Maor G
3. ESPE	Brussels	Sep. 2000	Maor G
4. Joint meeting of IBMS&ECTS	Madrid	June 2001	Maor G
5. ESPE	Ljubljana, slovenia	Sep. 2003	Phillip M
6. ESPE	Ljubljana, slovenia	Sep. 2003	Yanlonski G
7. ESPE	Ljubljana, slovenia	Sep. 2003	Maor G
8. ESPE	Ljubljana, slovenia	Sep. 2003	Maor G

14. SPECIAL PROFESSIONAL ACTIVITIES

a. Reviewed a manuscript for Odontology (2004).

b. Participated in the grant review committee on Endocrinology/Hard Tissues.- The Chief Scientist Office, Israeli Ministry of Health (2004).

c. A member of the review team for the abstracts for the 43rd (2005) Annual Meeting of the European Society for Paediatric Endocrinology (ESPE)

Gila Maor 10/2006

RESEARCH ACCOMPLISHMENTS

1. Metabolic acidosis-induced skeletal growth retardation.

Chronic metabolic acidosis (CMA) exhibit growth retardation accompanied by a decline of IGF-I and its receptors. However the etiology of the accompanied interference in skeletal growth is not fully understood. We have shown (31), in an ex vivo model for endochondral ossification subjected to acidic conditions, that chronic metabolic acidosis (CMA) directly induces growth retardation accompanied by a decline of IGF-I and its receptors. We have also shown (47) that PTH has ameliorative effects on the CMA-induced growth retardation. Lately we have explored the mechanisms underlining the beneficial effects of PTH on the acidic condyle; particularly the involvement of protein kinase A (PKA) and protein

kinase C (PKC). We have found that the CMA-induced growth retardation is probably attributed to proton-induced activation of PKA/CREB cellular cascade (BONE, 2004, submitted for publication).

2. The mechanism of estrogen-induced skeletal growth termination in the male's growth centers.

Gonadal steroid hormones are important for the normal pubertal growth spurt and for the subsequent closure of the growth plate (EGP) leading to a final growth arrest. It is not clear yet whether these effects are direct or indirect mediated. We have found that, testosterone has a direct, local, GH-independent effect on the EGP growth. It increases in a dose-dependent manner the abundance of IGF-1 receptor EGP (39). In addition, over-treatment with testosterone (dose or duration) accelerated condylar ossification resulting in an overall growth retardation (28). We have also found that estrogen (E) directly induces ossification on the expense accompanied by apoptosis (data not published yet). We now further exploring the mechanisms whereby E induces apoptosis-a study which will clarify the involvement of E in malignant processes.

3. The role of GLUT4 in IGF-I - mediated chondroenesis.

IGF-I, acting locally at the cartilaginous growth plates, is considered one of the major skeletal growth factors. In vivo studies have shown that IGF-I receptors (IGF-IR) are co-regulated with the major insulin dependent glucose transporter (GLUT)-GLUT4 (29). In further exploring the inter-relations between GLUT4 and IGF-I in modulating skeletal growth, we have shown that GLUT4-mediated glucose uptake in chondrocytes is merely modulated by IGF-I. Moreover, insulin-induced glucose uptake in the developing chondrocytes, is most likely mediated via IGF-IR. Using silencing GLUT4 mRNA approach, we could show that both IGF-I dependent glucose uptake and chondrogenesis activity are GLUT4 mediated (55). These observations contribute to better understanding the mechanisms of IGF-induced skeletal growth.

4. Development of spntaneously differentiating chondrocytes culture.

Great efforts have been devoted during the last decade aiming at developing a reliable model of primary chondrocytic tissue culture for both research and clinical applications. Separated chondrocytes tend to undergo dedifferentiation upon loosing their cell-cell and cell-matrix relationships. We have developed a novel -spontaneously differentiating chondrocytic culture model (44). This model based on a unique source for cartilage cells, a graduated enzymatic cell separation and specific culture medium, provides a reliable cartilage-mimicking tissue culture which is very useful for both basic science and clinical applications (the last is now being further developed in our Lab).

5. Osteoprotegerin (OPG) – a novel chondrogenic facror.

OPG was first identified as a decoy receptor of 'receptor-activated NF B' (RANKL) -the major osteoclast modulator. Hence, OPG counteracts the resorption activity of RANKL on bones (both factors belong to the TNF superfamily). We have shown that OPG is also expressed in cartilage forming cells. Moreover,

OPG modulates chondrogenesis rate. It induces cells proliferation, differentiation and maturation. Using IP approach we could also show specific binding sites for OPG on the cartilage cells. Thus, OPG may be considered as a novel local chondrogenic factor, acting, in a paracrine fashion. (these results have not been published yet).

6. The inter-relations between OPG and fibroblast growth factor (FGF)

FGF is known as for its cartilage growth arrest activities. Constitutive expression of its receptor FGFR3 causes achondroplasia- the most common congenital dwarfism. With collaboration with Dr Avner Yayon, we have started recently a novel research aiming at studying the protective/therapeutic effects of OPG on FGF induced impaired chondrogenesis. This work is based on genetically modified mice that display achondrplasia - like syndrome, and on our primary chondrocytes culture model over-treated with FGF variants.

Our preliminary results using both models seem quite promising

7. Developing a novel solution for treating joints' damages.

Damages in the articular cartilage surfacing the joints are one of the most acute/unsolved orthopedic problems. Being avascular and devoid of stem cells, adult cartilage does not renew itself. Moreover, untreated damages deteriorate, becoming secondary osteoarthritis lesions. Hence, a long-lasting rehabilitation solution should be based on biological replenishment of the lesion with cartilage producing cells. Yet, separated cartilage cells tend to undergo dedifferentiation upon re-culturing, producing mainly fibrocartilage – a sort of local scar tissue instead of the origin hyaline cartilage. We have developed a unique model, based on mandibular condyle derived cells (MCDC), of spontaneously differentiating cartilage cells producing genuine hyaline cartilage.

This MCDC cultures have been further developed into hyaline cartilage film, produced in vitro that can easily been transplanted into knee joint (goats') damaged articular cartilage. A further progression in this direction would be a development of a 'cartilage sticker' where by the cartilage film, attached to an inert biodegradable support, will be easily implantable through a minimal invasive procedure. The developing phase concentrates on searching the optimal adhesion conditions of the cartilage film to the membrane support while preserving the former normal developing in situ potential (after implantation). Expression of cartilage-specific genes, morphology and histology of the adhered cartilage film, will serve for assessing the potential of normal hyaline cartilage development under these conditions.

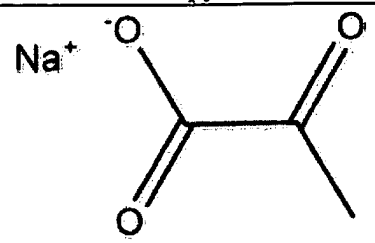
Sodium pyruvate

From Wikipedia, the free encyclopedia

Sodium pyruvate is commonly added to cell culture media as an additional source of energy, but may also have protective effects against hydrogen peroxide. This was reported by Giandomenico *et al.*[1] and has been confirmed by several independent groups.

References

1. ^ Giandomenico AR, Cerniglia GE, Biaglow JE, Stevens CW, Koch CJ (1997). "The importance of sodium pyruvate in assessing damage produced by hydrogen peroxide.". *Free Radic Biol Med* **23** (3): 426-34. doi:10.1016/S0891-5849(97)00113-5. PMID 9214579.

Sodium pyruvate	
 sodium pyruvate	
Other names	α-ketopropionic acid sodium salt 2-oxopropanoic acid sodium salt Pyruvic acid sodium salt
Identifiers	
CAS number	113-24-6
Properties	
Molecular formula	$C_3H_3NaO_3$
Molar mass	110.0 g/mol
Solubility in other solvents	100 mg/mL
Except where noted otherwise, data are given for materials in their standard state (at 25 °C, 100 kPa) Infobox references	

Hypertrophic Chondrocytes Undergo Further Differentiation in Culture

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Abstract. Conditions have been defined for promoting growth and differentiation of hypertrophic chondrocytes obtained in culture starting from chick embryo tibiae. Hypertrophic chondrocytes, grown in suspension culture as described (Castagnola P., G. Moro, F. Descalzi Cancedda, and R. Cancedda. 1986. *J. Cell Biol.* 102:2310–2317), when they reached the stage of single cells, were transferred to substrate-dependent culture conditions in the presence of ascorbic acid. Cells showed a change in morphology, became more elongated and flattened, expressed alkaline phosphatase, and eventually mineralized. Type II and X collagen synthesis was halted and replaced by type I collagen synthesis. In addition the cells started to produce and to secrete in large amount a protein with an apparent molecular mass of 82 KD in reducing conditions and 63 KD in unreducing conditions. This protein is soluble in acidic solutions, does not contain collagenous domains, and is glycosylated. The Ch21 protein, a marker of hypertrophic chondrocytes and bone cells, was synthesized throughout the culture. We have defined this additional differentiation stage as an osteoblast-like stage. Calcium deposition in the extracellular matrix occurred regardless of the addition of β glycerophosphate to the culture medium. Comparable results were obtained both when the cells were plated at low density and when they were already at confluence and maintained in culture without passaging up to 50 d. When retinoic acid was added to the hypertrophic chondrocyte culture between day 1 and day 5 the maturation of the cells to the osteoblast-like stage was highly accelerated. The switch in the collagen secretion was already observed after 2 d and the production of the 63-kD protein after 3 d. Mineralization was observed after 15–20 d.

Long bone organogenesis occurs in the embryo by endochondral ossification from undifferentiated mesenchyme. During the early stages of development, mesenchymal cells in the limb buds condense to form a core of differentiated chondrocytes; osteogenesis starts at the periphery of the cartilage core, which is subsequently invaded by blood vessels and replaced by bone marrow and trabecular bone. After birth, similar events take place in the long bone growth plate and at the bone fracture sites. Bone formation and remodeling have been extensively investigated, starting from pioneering work describing the morphological and biochemical changes occurring during early bone formation to more recent studies aimed at the elucidations of the cellular and molecular mechanisms involved (7, 22, 34). It is widely agreed that cells present in a continuous collar surrounding, but separated from the cartilage rudiment, give rise to osteoblasts, i.e., cells responsible for the synthesis and mineralization of the osteoid extracellular matrix. In the past, occasionally and recently more frequently, it has been postulated that growth plate hypertrophic chondrocytes might also contribute to the formation of a bone matrix, since in some organ cultures these cells start to express bone markers. During culture of mouse mandibular condyles, the expression of type I collagen, osteonectin, alkaline phosphatase, osteopontin, and osteocalcin by mature chondrocytes was detected by in situ hybridization (38). A morphological study has been recently published consistent with the idea that in condylar cartilage of rat mandible hypertrophic chondrocytes can become bone (48). Conversion of mature chondrocytes into osteogenic cells was also observed in Meckel's cartilage from rat in ocular culture (30).

We have described that hypertrophic chondrocytes in suspension culture undergo terminal differentiation and express large amount of type X collagen (8), a marker specific for hypertrophic cartilage (6, 31, 32), and Ch21, a protein belonging to the superfamily of proteins binding small hydrophobic molecules that is expressed both by cartilage and bone (11, 12, 24).

In the present investigation we show that when hypertrophic chondrocytes isolated as single cells after 3 wk in suspension culture are transferred to anchorage-dependent culture conditions in the presence of ascorbic acid, they undergo profound changes in their morphology and biosynthetic activity suggesting a further maturation to an osteoblast-like stage. Cells acquire an elongated or star shaped morphology, start to express alkaline phosphatase, and to reorganize their extracellular matrix by switching from the synthesis of the cartilage specific type II and type X collagens to the synthesis of type I collagen. At the same time, cells express and secrete a large quantity of a glycoprotein with an apparent molecular mass of 63 kD in unreducing conditions. Eventually, on the newly formed matrix calcium minerals are deposited.

In addition, we report that when retinoic acid, a compound well known for its capability to interfere with skeletal development in vivo and with cartilage and bone cells differentiation in vitro, is added to the hypertrophic chondrocyte culture between day 1 and day 5, the conversion of chondrocytes from stage II to the osteoblast-like stage is highly accelerated. The switch in the collagen secretion is already observed after 2 d and the production of the 63k protein after 3 d. Mineralization is observed after 15–20 d.

Materials and Methods

Cell Cultures

Dedifferentiated and hypertrophic chondrocytes were obtained from 6-d chick embryo tibiae. Cells derived from the cartilaginous bone were expanded as adherent dedifferentiated cells for 2 wk and then transferred in suspension culture for 3–4 wk until a homogeneous population of single isolated hypertrophic chondrocytes was obtained (8). Hypertrophic chondrocytes were filtered through a nylon filter Nitex 42 μm mesh in order to avoid any contamination of cells still aggregated, digested with hyaluronidase (1 mg/ml), and plated either at low density (5×10^4 cells in 100-mm dish) or at confluence (2×10^5 in a 35-mm dish) in Coon's modified F12 culture medium (1) containing 10% FCS. After 3 d the medium was supplemented with 100 μg/ml ascorbate and 10 mM β-glycerophosphate (complete medium). The medium was changed every other day without cell passaging. Parallel cultures grown without ascorbic acid were used as control. When indicated, retinoic acid was added to the culture medium at 1 μM concentration. Retinoic acid from a freshly prepared solution was daily added to the medium from day 1 to day 5 of the experiment and afterwards cultures were maintained in complete medium.

Cell Labeling and Protein Analysis

Cells were labeled with ^{35}S methionine as described (11). When indicated, tunicamycin (2 μg/ml) was added to the culture medium during methionine starvation and the 2-h labeling period. Aliquots of culture media or cell lysates were run for protein analysis on SDS-PAGE in unreducing and reducing conditions as described (3). Except when indicated polyacrylamide gel concentration was 12.5%. When indicated, samples were digested with bacterial collagenase (20 μg/ml) in 0.05 M Tris-HCl, pH 7.6, 0.01 M $CaCl_2$ at 37°C for 90 min before electrophoresis. In some experiments, the samples were dialyzed for 16 h at 4°C in 0.5 N acetic acid, digested with pepsin (100 μg/ml) overnight and lyophilized before analysis. Immunoprecipitation of specific proteins was performed as previously described (11).

Histochemistry

Alcian blue staining (Chroma, Stuttgart, Germany) specific for cartilage proteoglycans was performed at pH 1 (4). Alkaline phosphatase activity was

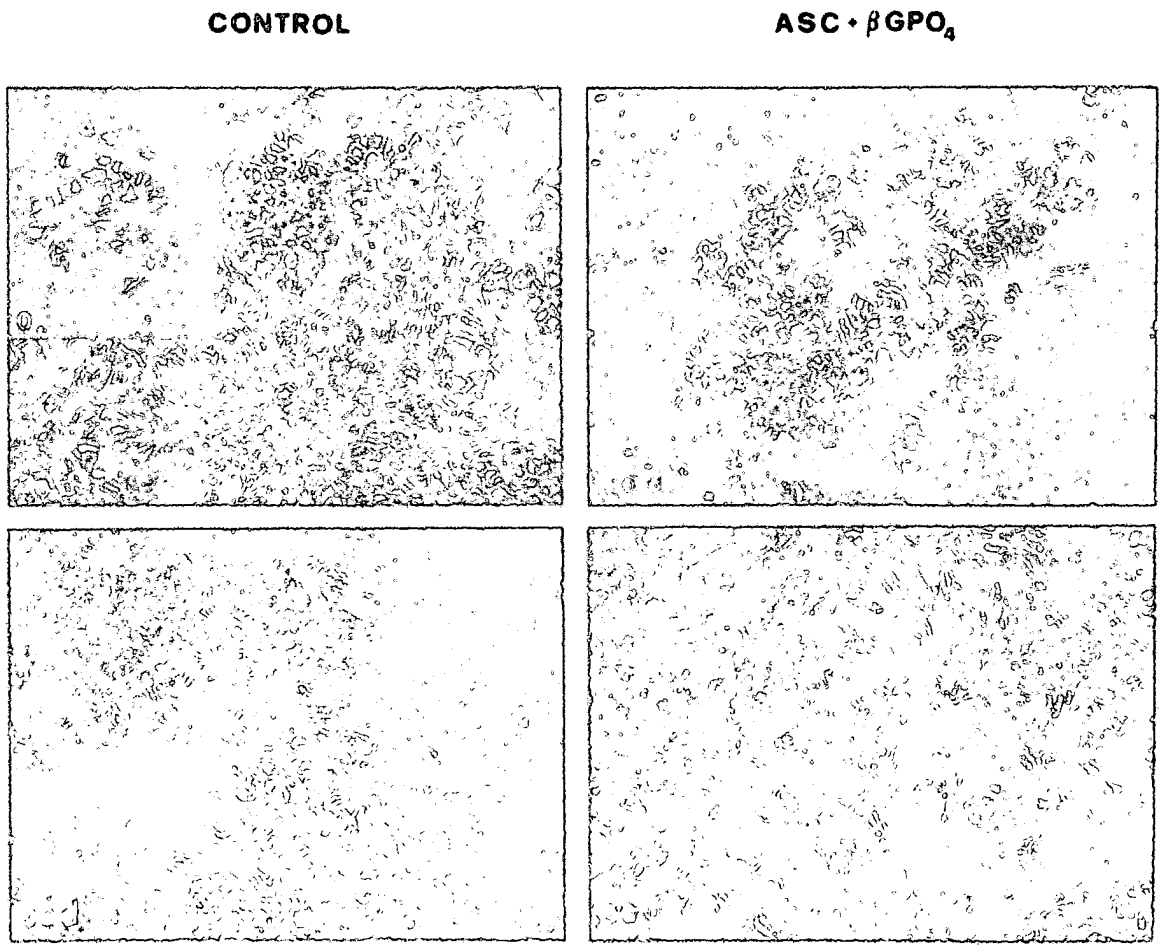

Figure 1. Cell morphology of hypertrophic chondrocytes plated at low density. Cells were maintained in Coon's modified F12 medium (*control*) or in the same medium supplemented with 100 μg/ml ascorbic acid and 10 mM β glycerophosphate. Numbers refer to days in culture. The insert "0" refers to the starting cell population. Bar, 100 μm.

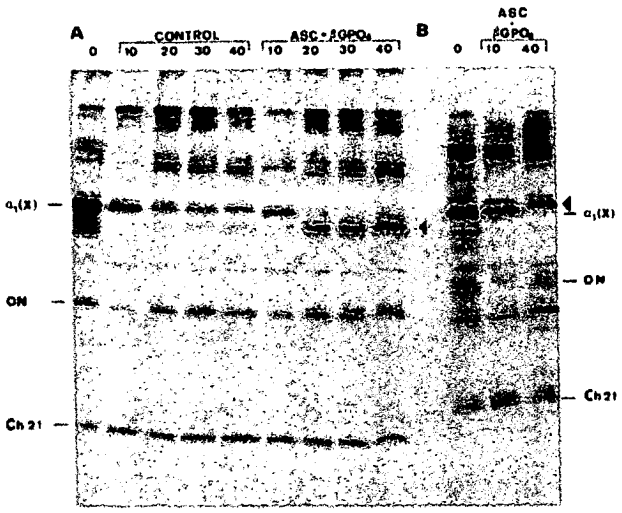

Figure 2. Proteins secreted by cells plated at low density. Cells were maintained in Coon's modified F12 medium (*control*) or in the same medium supplemented with 100 μg/ml ascorbic acid and 10 mM β glycerophosphate. Numbers refer to days in culture; "0" refers to suspension culture of hypertrophic chondrocytes at the time they were plated. Proteins were analyzed in unreducing (*A*) and reducing (*B*) conditions. Arrowheads refer to the protein of 63 KD in unreducing conditions and 82 KD in reducing conditions described in the text. Other differentiation markers: type X collagen, osteonectin (ON), and Ch21 were identified based on their electrophoretic migration and their immunoprecipitation by specific antibodies (not shown).

determined using the histological kit 86 by Sigma Chemical Co. (St. Louis, MO) and mineralization was detected by von Kossa staining (4).

Results

Protein Synthesis by Plated Hypertrophic Chondrocytes Maintained in Supplemented and Unsupplemented Control Medium

Hypertrophic chondrocytes were recovered from a 3–4-wk suspension culture, filtered through a Nitex filter in order to eliminate any possible residual cell aggregate, digested with hyaluronidase, and plated at a low density both in the presence and in the absence of ascorbic acid and β glycerophosphate. In these conditions, hypertrophic chondrocytes remained replicative, although at a low rate, and reached confluence in 10–15 d. Fig. 1 shows the cell morphology at different time of culture in both conditions. We constantly observed that after 4–5 wk of culture the cells maintained in the presence of ascorbic acid and β glycerophosphate, but not the control cells, presented a frank fibroblastic morphology. The different morphology was even more evident when the hypertrophic chondrocytes were plated at confluence (not shown).

The pattern of proteins secreted by the cells during culture is shown in Fig. 2. It is evident from Fig. 2 *A* that major differences exist between proteins secreted by cells maintained in the presence of ascorbic acid and β glycerophosphate and proteins secreted by control cells. In particular, the cells maintained in the supplemented medium stopped releasing type X collagen in the medium between day 10 and day 20 of culture, while control cells continued to secrete the same molecule, although at a lower level, until at least the 40th day of culture. The disappearance of type X collagen from the culture medium of the cells maintained in the supplemented medium was contemporary to the appearance of a new protein with an apparent molecular mass of 63 kD (Fig. 2, *arrowhead* in *A*). The same protein was barely detectable in the culture medium of control cells only at late times. It must be noted that when polyacrylamide gel electrophoresis was performed in reducing conditions (Fig. 2 *B*) this protein had an electrophoretic mobility very similar to the mobility of type X collagen making it difficult to discriminate between the two proteins. When the production of collagens was specifically investigated by the analysis of the pepsin resistent domains and their immunoprecipitation by specific antibodies, we observed that the arrest in type X collagen synthesis was contemporary to the switch from the synthesis of type II collagen to the synthesis of type I collagen (Fig. 3). The analysis of the collagens that remained associated with the cell layers confirmed the observations made by analyzing the proteins released into the media (not shown).

In some experiments, hypertrophic chondrocytes were plated directly at confluence. In the presence of ascorbic acid the production of the 63k protein and the expected changes in the collagen secretion occurred regardless of the different concentration at which the cells were plated (see Fig. 8 *A*).

A preliminary characterization of the 63k protein secreted by the cells maintained in supplemented medium revealed that this protein is not a collagen, since it was completely digested by pepsin treatment (Fig. 3, *A* and *B*) and its electrophoretic migration was not affected by collagenase digestion (Fig. 4 *A*), but it is glycosylated (Fig. 4 *B*). The 63k protein was not synthesized by the starting population of dedifferentiated cells, from which the hypertrophic chondrocytes were obtained, both in supplemented and control medium (Fig. 4 *C*). It must be noted that the dedifferentiated cells were only labeled 5 d after reaching confluence; in fact, the dedifferentiated cells, at variance with the cells derived from the hypertrophic chondrocytes, started to detach from the culture dish a few days after becoming confluent.

Extracellular Matrix Mineralization

Cells maintained in culture in supplemented and in control medium were Alcian stained at low pH in order to search for the presence of cartilage specific proteoglycans in the extracellular matrix. We observed a progressive decrease of Alcian positive extracellular matrix in cultures made in the presence of ascorbic acid and β glycerophosphate. By the fourth week in culture except for a few spotted areas all the matrix was negative; at the same time the cell extracellular matrix of control cultures was still highly positive (Fig. 5).

Cultures maintained in supplemented medium were also examined for alkaline phosphatase activity and deposition of calcium minerals by histological staining. Cells with alkaline phosphatase activity were already present at 10 d. The cells with maximal level of activity had both a stellate and a fibroblastic morphology (Fig. 6 *A*); in the same dish and in cultures maintained for the same time in control medium, some cells presenting the characteristic chondrocyte polygonal morphology also had detectable level of alkaline phosphatase activity. The first histologically detectable calcification was observed in the culture maintained in the presence

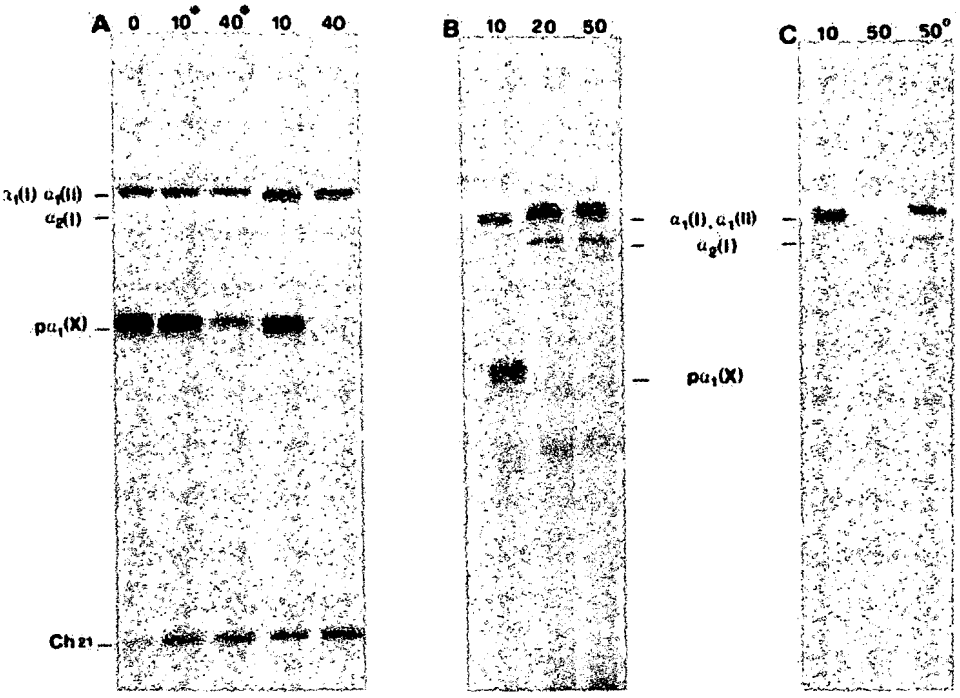

Figure 3. Collagens secreted by cells plated at low density. Aliquots of media from the same experiment of Fig. 2 were pepsin digested and analyzed on 10% polyacrylamide gel. Numbers refer to days in culture. All media were from cells maintained in the presence of ascorbic acid and β glycerophosphate, but the ones marked with asterisks were from cells maintained in F12 without additions (*control*). p(α_1)X indicates the collagenous domain of pepsinized type X collagen. Samples in *C* are the same samples as in *B* after immunoprecipitation with specific antibodies against type II collagen (first two lanes) and against type I collagen (symbol ° in third lane).

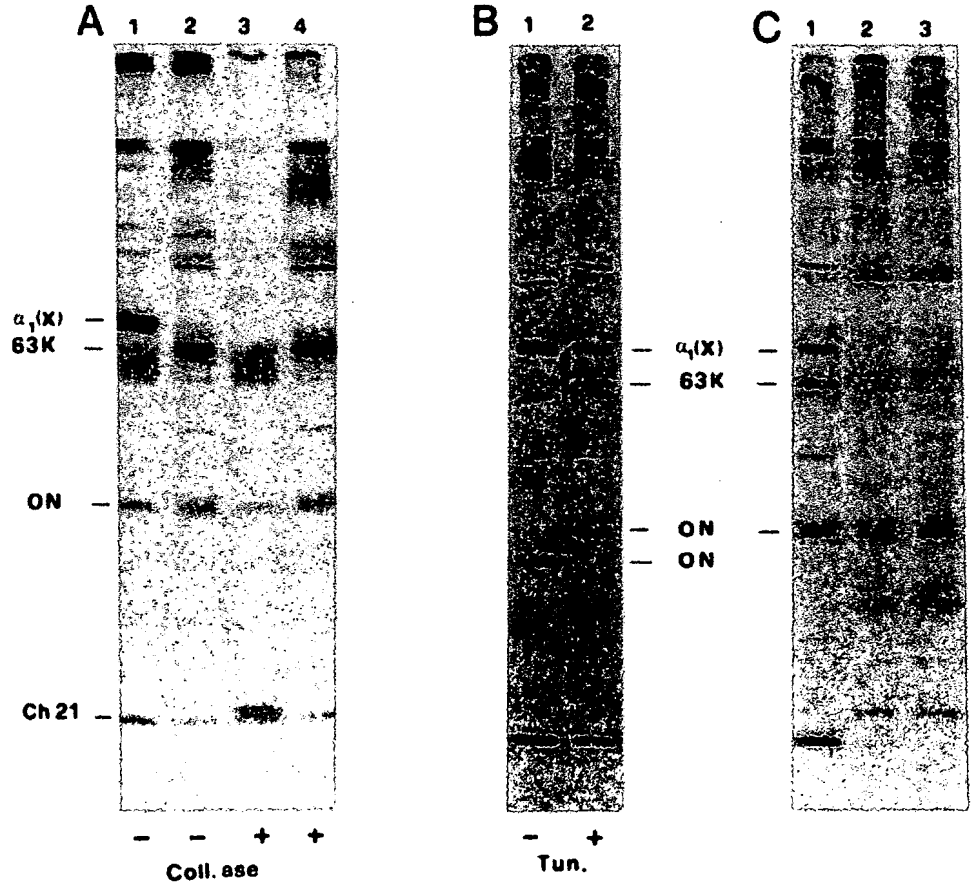

Figure 4. Characterization of the 63-KD protein. (*A*) Proteins secreted by control hypertrophic chondrocytes in suspension (lanes *1* and *3*) and by hypertrophic chondrocytes plated and cultured in the presence of ascorbic acid and β glycerophosphate (lanes *2* and *4*) were analyzed before and after collagenase digestion. (*B*) Proteins secreted by hypertrophic chondrocytes grown as adherent cells in supplemented medium and labeled in the presence or in the absence of tunicamycin. (*C*) Proteins secreted by hypertrophic chondrocytes grown as adherent cells in supplemented medium for 5 d (lane *1*) were analyzed in parallel with proteins secreted by dedifferentiated chondrocytes grown for 5 d in supplemented (lane *2*) and nonsupplemented (lane *3*) medium. ON indicates osteonectin.

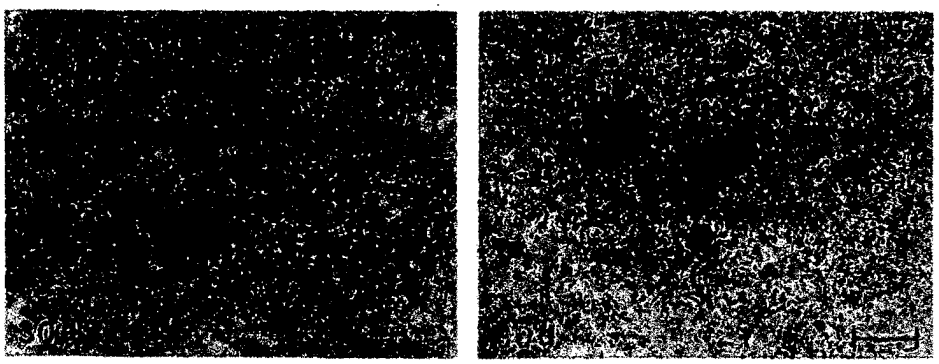

Figure 5. Alcian staining of cultured cells. Hypertrophic chondrocytes were stained after 30 d from plating in supplemented and not supplemented medium. Bar, 100 μm.

of ascorbic acid and β glycerophosphate after ~40–50 d. The mineralization extended throughout the culture and it reached its highest peak after ~7–8 wk (Fig. 6 *B*). The deposition of calcium mineral was enhanced by the high cell density.

It has been suggested that the presence of mM concentrations of β glycerophosphate in the culture medium may promote calcium mineral deposition per se independently of the differentiation stage of the cells in culture. To rule out a direct role of the glycerophosphate on the mineralization occurring in our cell system, some cultures were performed in the presence of ascorbic acid, but omitting the addition of β glycerophosphate to the medium. No major differences were observed in the timing and nature of stellate and fibroblastic morphology development. No major differences were observed when proteins secreted during culture were analyzed and alkaline phosphatase activity and mineralization were determined by histological staining (not shown).

Retinoic Acid Plays a Major Role in Promoting Maturation of Hypertrophic Chondrocytes to Osteoblast-like Cells

Hypertrophic chondrocytes were plated at confluence and maintained in culture for ~4 wk in the presence of ascorbic acid. Phase-contrast micrographs were taken both of cultures that were supplemented with 1 μM retinoic acid during the first 5 d and of control cultures (Fig. 7, *A* and *B*). In the cultures supplemented with retinoic acid, the cells acquired a fibroblastic morphology already during retinoic acid treatment. Histochemical stainings revealed absence of cartilage-specific proteoglycans after 5 d only in retinoic acid–treated cultures (Fig. 7 *C*). Alkaline phosphatase–positive cells were observed after 15 d in both cultures, although the morphology of positive cells varied. In retinoic acid–treated cultures, positive cells always had a fibroblastic or stellate shape, while in control cultures there were also positive cells presenting a more regular polygonal morphology (not shown). Calcium mineral deposition was observed after 20 d in the retinoic acid–treated cultures, but not in control cultures (Fig. 7 *D*).

Proteins secreted by the cells were analyzed from day 1 to day 20. Fig. 8 shows the proteins secreted by hypertrophic chondrocytes cultured in: (*a*) Coon's modified F12 medium supplemented with FCS and containing also ascorbic acid and β glycerophosphate (Fig. 8 *A*) (*b*) the same medium with the further addition of retinoic acid during the first 5 d of culture (Fig. 8 *B*). Disappearance of type X collagen and production of the 63k glycoprotein was observed in retinoic acid–treated cultures between the first and the fifth day (see also Fig 9 *B*), while in control cultures not supplemented with retinoic acid, type X collagen was still present, although at a low level, after 20 d and the 63k protein continuously increased during culture. The high molecular weight interstitial collagens were better analyzed on pepsin digested

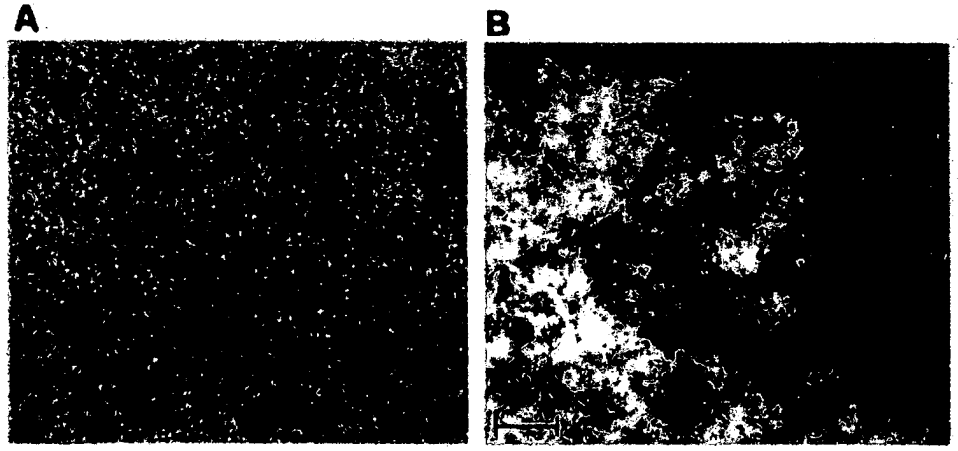

Figure 6. Histochemical staining of cultured cells. Hypertrophic chondrocytes maintained as adherent cells in supplemented medium were stained for alkaline phosphatase after 30 d (*A*) and for mineral deposition by von Kossa staining after 50 d (*B*). Bar, 100 μm.

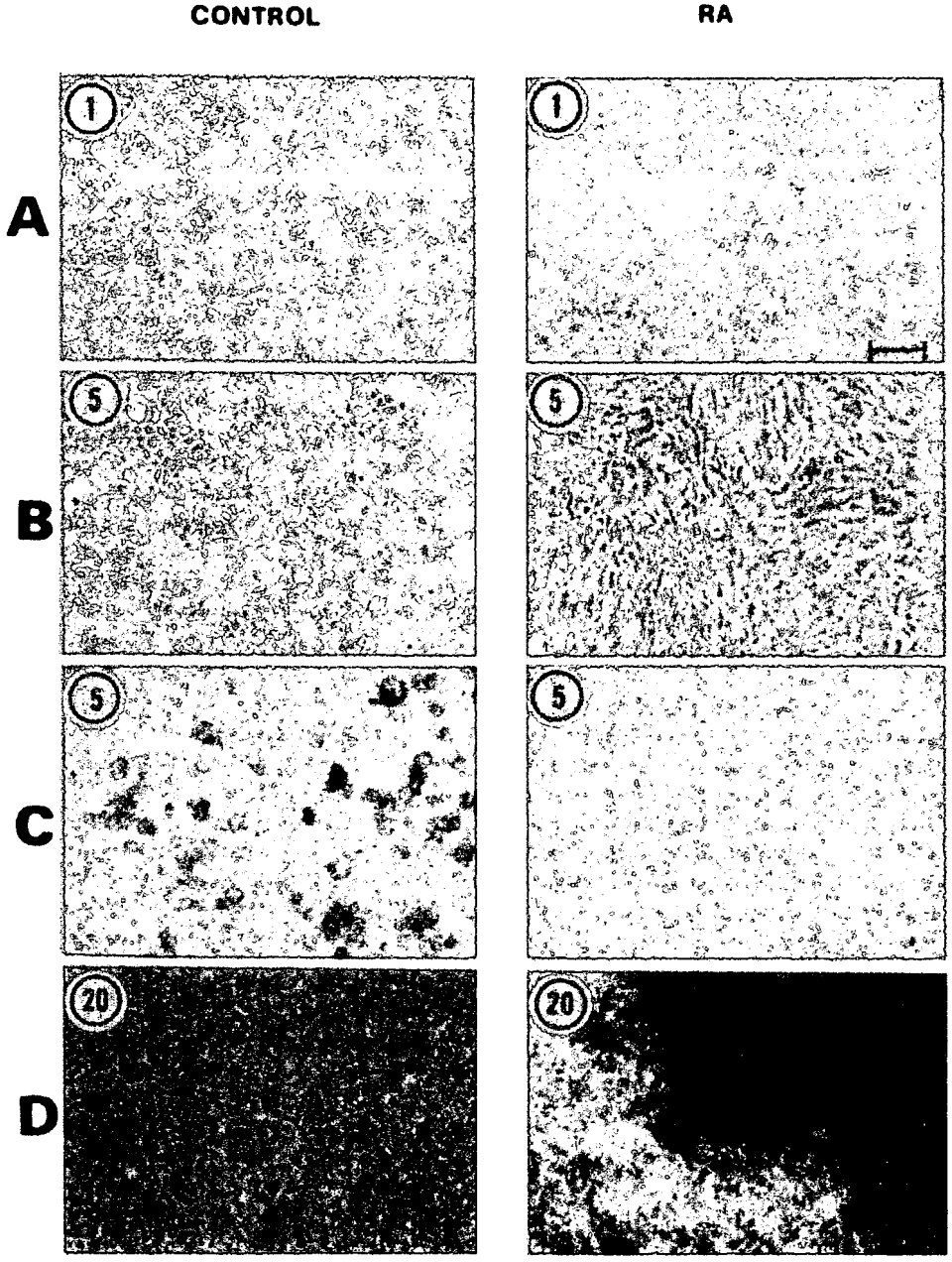

Figure 7. Effect of retinoic acid on cell morphology and histochemical staining of plated hypertrophic chondrocytes. Hypertrophic chondrocytes were plated at confluence and maintained in supplemented medium. Retinoic acid (*RA*) was added during the initial 5 d of culture. In control cultures the addition of retinoic acid was omitted. Phase-contrast pictures were taken 1 and 5 d after the addition of retinoic acid (*A* and *B*). Parallel cultures were Alcian stained at day 5 (*C*) and von Kossa stained after 20 d (*D*). Bar, 100 μm.

samples (Fig. 9). Switch from type II to type I collagen was investigated daily from day 1 to day 5 and it was observed that the collagen switch occurred in retinoic acid–treated cells between day 2 and day 3 (*B*). Type I collagen was the only collagen detectable between day 15 and day 25, when mineralization occurred.

Discussion

The relevance of the extracellular matrix in determining the cell microenvironment and the importance of the cell–extracellular matrix interactions in promoting and maintaining a differentiated phenotype is widely acknowledged. Several authors, including us, have shown that environmental conditions strongly influence chondrocyte development in cell culture systems and may promote cell hypertrophy starting from cartilage cells of different origin (5, 8–10, 14, 16, 36, 37, 39, 40, 45). Cultures within collagen gels, long-term secondary cultures on plastic, and suspension culture on agarose have been used to obtain chondrocytes at different development stages. In particular, by culturing dedifferentiated chondrocytes on agarose we were able to obtain a homogeneous population of single cells with hypertrophic chondrocyte traits with regard to hypertrophy, type X collagen production (9), and loss of proliferation capacity (15). Here we reported that when these hypertrophic chondrocytes are kept as adherent cells in the presence of ascorbic acid, a condition that allows a correct stable assembly of the collagen molecules, they become elongated or star shaped, express alkaline phosphatase, and modify their extracellular matrix by stopping synthesis

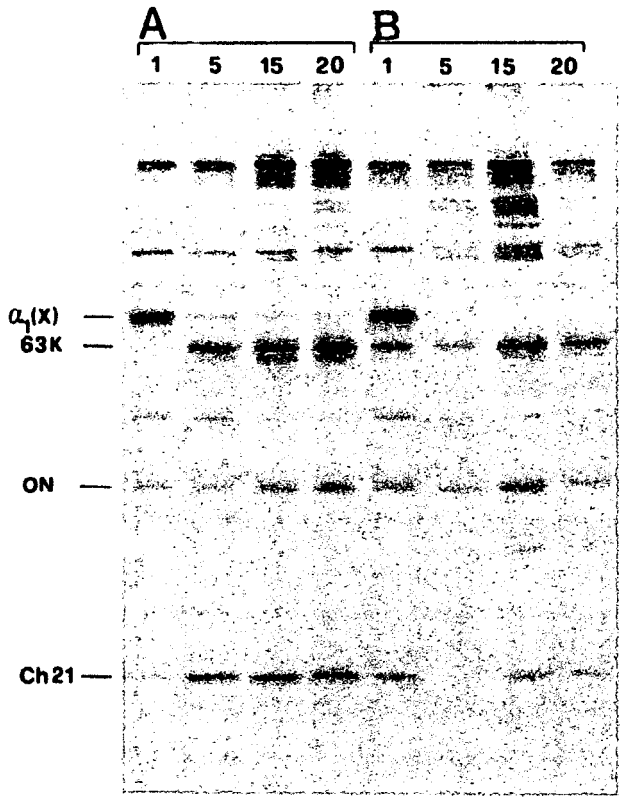

Figure 8. Proteins secreted by cells treated with retinoic acid. Cells were maintained in Coon's modified F12 medium supplemented with 100 μg/ml ascorbic acid and 10 mM β glycerophosphate (*A*) or in the supplemented medium with the further addition of retinoic acid from day 1 to day 5 (*B*). Numbers refer to days in culture. Proteins were analyzed in unreducing conditions. ON indicates osteonectin.

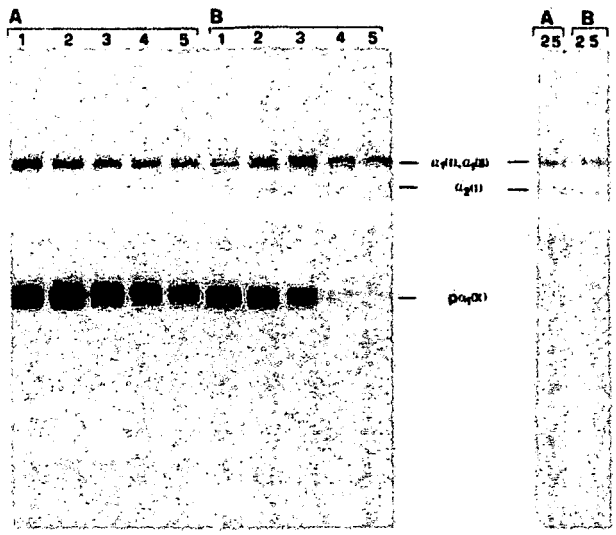

Figure 9. Collagens secreted by cells treated with retinoic acid. Cells were maintained in Coon's modified F12 medium supplemented with 100 μg/ml ascorbic acid and 10 mM β glycerophosphate (*A*) or in the same medium with the further addition of retinoic acid from day 1 to day 5 (*B*). Numbers refer to days in culture. Lanes A_{25} and B_{25} were from the same cultures but were analyzed on a separated gel. At 25 d the only collagen synthesized was type 1 collagen. Aliquots of media were pepsin digested and analyzed on 10% polyacrylamide gel in unreducing conditions.

of cartilage-specific proteoglycans and type II and type X collagens and by initiating synthesis of type I collagen. The Ch21 protein is produced by these cells at all differentiation stages, while the synthesis and secretion of a 63k glycoprotein initiates during the culture and is highly expressed by the osteoblast-like cells. Studies to further characterize this 63-k glycoprotein are in progress in our laboratory. Eventually in the modified matrix mineralization occurs. The addition of β glycerophosphate results in a more rapid mineral deposition but is not an absolute requirement for mineralization. Certainly other factors modulate extracellular matrix mineralization. We observed that the same cells maintained in culture media containing different batches of FCS underwent mineralization at significantly different time after plating (our unpublished results). When the chondrocytes were kept in suspension culture in the absence of ascorbic acid we did not observe the phenotypic transition (not shown).

It is interesting to note that hypertrophic chondrocytes plated in culture dishes after enzymatic removal of their extracellular matrix attach to the dishes and resume proliferation. Cells plated at very low density reach confluence in 10–15 d. Nevertheless, differentiation of hypertrophic chondrocytes to osteoblast-like cells occurs also when cells are directly plated at confluence, a condition where cell multiplication is impaired. In agreement with the knowledge that an inverse relationship exists between cell proliferation and expression of differentiation markers, maximum of differentiation was observed in confluent cultures.

In vivo the expression of genes characteristic of osteogenic differentiation have been reported in cells in the zone of hypertrophic chondrocytes and the possibility that mature chondrocytes start to sequentially activate osteogenic marker genes has been considered (30, 38, 48). In this report we have shown that the possibility exists that hypertrophic chondrocytes, depending on their microenvironment, undergo further differentiation and express markers common to bone cells. It is worth noting that the boundary between cartilage and bone in the growth plate of the long bones is characterized by profound rearrangement and modification of the extracellular matrix. Based on our in vitro findings, it is tempting to suggest an active role of hypertrophic chondrocytes during in vivo remodeling of hypertrophic cartilage into bone, but further studies are necessary to prove this hypothesis.

The vitamin A derivative retinoic acid is widely known to affect the differentiation state of a variety of tissues including cartilage. In early studies it has been shown that this compound induces facial and cranial malformations in developing embryos (21, 41). More recent investigations suggest that retinoic acid is actually involved in normal cartilage development. It has been reported that chick embryo limb buds contain endogenous retinoic acid forming a concentration gradient from the anterior to the posterior region of the limb bud (13, 42). Retinoic acid–soaked beads induce striking digit pattern duplication when locally applied to the developing limb bud and mimic the action of a graft of the posterior margin region, named zone of polarizing activity, where a group of mesenchymal cells are localized which specify the

pattern of mesenchymal tissues on the antero-posterior axis (25, 27, 43, 46).

The effect of retinoic acid on the chondrocytes "in vitro" has been extensively studied. Retinoids affect specific characteristics of cultured chondrocytes including cell shape and glycosaminoglycan and protein synthesis. Its mechanism of action is unknown but there are evidences that retinoids cause changes in the synthesis of the extracellular matrix components without any indication of toxic effects (2, 18, 19, 20). In the early phases of chondrocyte differentiation, as it has been reported by several authors (17, 23, 28, 33, 35, 44, 47), retinoic acid either inhibits or induces chondrogenesis. At later stages of chondrocyte differentiation, retinoic acid stimulates chondrogenesis and promotes maturation of chondrocytes to stage II (hypertrophic, type X producing) (26–29) and osteoblast-like cells (type I producing, mineralizing) (this report). It is therefore evident that differentiating chondrocytes exhibit different responses to exogenous retinoic acid depending upon their differentiation stage.

Studies aimed at further investigating the organization of the extracellular matrix and the nature of mineralization in osteoblast-like cell culture both in the presence and in the absence of retinoic acid are currently under investigation.

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Collagenase and Gelatinase Production by Calcifying Growth Plate Chondrocytes

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The regulation of cartilage resorption is important both in cartilage pathophysiology and angiogenesis. Previous work has identified a link between calcification and activation of collagenolysis. The aim here was to test whether production of collagenase itself is also calcification-dependent, using a high-density growth *plate chondrocyte culture model of calcification*. Ultrastructural studies indicated that calcification occurred around large hypertrophic cells. There was no indication of phagocytosis of crystals even by cells lying next to mineral aggregates, although remodeling of the organic matrix by cell processes was evident. Release of collagenase activity increased dramatically between 24 and 48 h postcalcification, from low or undetectable basal levels. In contrast, gelatinase production was not calcification-dependent. Collagenase was released almost entirely in the latent form, being a consequence of increased protein synthesis rather than activation of existing enzyme. This linkage of calcification with latent collagenase production represents part of a coordinated remodeling of both collagenous and mineral components of the matrix which may also extend, *in vivo*, to the control of microvascular invasion and resorption.

INTRODUCTION

During the last stages of differentiation in the mammalian growth plate, chondrocytes undergo hypertrophy and remodel their surrounding matrix [1, 2]. At this stage, remodeling appears to involve degradation and subsequent growth of the cartilage matrix [1, 3, 4]. Later remodeling involves a polarized calcification of the cartilage (i.e., limited to the longitudinal septae while sparing the transverse septae) and final resorption during neovascularization and ossification [2]. The resorption stage is thought to involve collagenolysis, adjacent to invading endothelial cells [5–8]. Certainly, endothelial cells can produce matrix-degrading proteases capable of this resorption [9]. However, hypertrophic chondrocytes themselves produce collagenase [4, 10, 11] and this has led to the suggestion that it is the chondrocytes which initiate final cartilage resorption [12]. Consequently, if both remodeling and resorption are mediated by activation of the same proteolytic system, it is important to determine how that system is initiated during the earlier, remodeling stage.

Cartilage matrix lysis is now thought to be initiated by the release of latent matrix metalloproteinases (MMPs), including collagenase [13]. Once activated, these enzymes depolymerize and cleave collagen and noncollagenous proteins. However, MMPs are not readily activated in cartilage and once activated they are rapidly inhibited by tissue inhibitors of metalloproteinase (TIMP). An additional level of regulation has been identified in the form of endothelial cell-stimulating angiogenesis factor (ESAF). ESAF was initially identified on the basis of its potent angiogenic activity, apparently acting by stimulation of matrix lysis and endothelial cell invasion [6, 7, 14]. This low-molecular-weight compound is capable of activating latent collagenase [14] and reactivating TIMP–collagenase complex to give active enzyme [15, 16]. It remains the only known, naturally occurring activator of the TIMP–collagenase complex.

Growth plate chondrocytes *in vivo* and *in vitro* produce ESAF and this has been shown, *in vitro*, to be stimulated by calcification of the matrix [7, 17]. *In vivo*, hypertrophic chondrocytes also produce collagenase [4, 10, 11], which may be involved in matrix growth associated with hypertrophy [1, 3]. Since production of the enzyme activator (ESAF) is related to matrix calcification it seems reasonable to question whether enzyme production is similarly calcification-dependent. Increased production of both the enzyme and its activator, ESAF, following the same external stimulus (i.e., calcification) would suggest that this represents a coordinated cell response. The aim of the present study was to test this, on isolated growth plate chondrocytes, by monitoring the production of collagenase following cal-

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cification. Production was also compared with that of another matrix metalloproteinase, gelatinase, and correlated with structural changes in the cultures.

MATERIALS AND METHODS

Calcium β-glycerophosphate (CaBGP), cycloheximide, and clostridial peptidase A (collagenase type I; EC 3.4.24.3) were from Sigma Chemicals (Poole, Dorset, UK). Medium E199, with L-glutamine was from Gibco (Paisley, Scotland). [^{3}H]Acetic anhydride was from Amersham International (Amersham, Bucks, UK). Sandy lop rabbits, at 6 weeks (approx 1 kg body wt) were used to prepare growth plate chondrocytes. Acid-soluble type I collagen was purified from fetal calf skin [18] while type II collagen was from pepsin-solubilized fetal calf cartilage reprecipitated at acid and neutral pH [19]. Endothelial cell growth supplement (bovine pituitary extract, rich in fibroblast and other growth factor [20]), human recombinant basic fibroblast growth factor (b-FGF), and heparin sulfate were from Sigma Chemicals.

High-density primary cultures of rabbit growth plate chondrocytes (micromass and large scale, based on initial inoculum) were prepared as described previously [7, 21]. Briefly, rabbit long bone growth plates were digested with clostridial collagenase to release a cell suspension. Following repeated centrifugation and resuspension, cleaned cells were grown at an initial inoculum of 200,000 cells for each micromass dot culture, using either two or four micromass dots on each 35-mm petri dish, for 14 to 21 days in E199 medium (1 ml/dish), containing 42 units/ml gentamycin, 150 μg/ml ascorbate, and 10% fetal calf serum, changed every 48 or 72 h. Micromass cultures of rabbit articular cartilage chondrocytes were prepared in the same way. Alternatively, larger scale, megamass, high-density cultures were used, where specified, to produce greater quantities of enzyme. These used an initial inoculum of 2×10^6 chondrocytes, cultured in round-bottomed universal plastic tubes, with 5 ml of the same medium. Prior to addition of test media, cultures were rinsed in serum-free medium and incubated in fresh serum-free medium for a pretreatment period. All subsequent treatments were carried out in serum-free medium plus experimental additives (see below).

Conditioned media were assayed for collagenase and gelatinase as described previously [14, 22] modified from Gislow and McBride [23]. Enzyme activity was determined as micrograms of substrate (gelatin or collagen) degraded per hour and normally expressed per culture. Conditioned media were assayed in the absence and presence of aminophenylmercuric acetate (APMA) to determine both active enzyme and total (active + latent) enzyme activities.

For light and electron microscopy, cultures were fixed in 2% glutaraldehyde, 0.1 *M* sodium cacodylate buffer, pH 7.2, for 2 h at 4°C and washed in the same buffer. Postfixation was in 1% osmium tetroxide in 0.1 *M* sodium cacodylate buffer, pH 7.2, at room temperature for 1 h and washed in two changes of 0.1 *M* sodium cacodylate buffer. Cells were dehydrated in their respective culture dishes through a graded series of alcohols and three changes of absolute ethanol. Cell layers were removed from culture dishes by treatment with propylene oxide [24]. Absolute ethanol was replaced with propylene oxide and after a few seconds with gentle agitation adherent cell layers were released from their culture dishes and transferred to fresh propylene oxide in glass containers. Cell layers were put directly into Spurr's resin, infiltrated for 2 h, embedded, and cured (−70°C for 18 h). One-micrometer sections were cut for light microscopy (LKB ultratome) and stained by the Humphrey's trichrome method [25]. Ultrathin sections were cut at 90 nm, stained with 2% uranyl acetate (10 min) and Reynold's lead citrate (10 min), and viewed in a Philips CM12 electron microscope at 80 kV. X-ray probe microanalysis, to confirm the mature of calcific deposits, used 120-nm unstained sections in the same CM12 instrument at a take-off angle of 40°.

Experimental additions to cultures included 4 m*M* CaBGP to initiate calcification [7, 21], 2 m*M* levamisole to inhibit matrix calcification [17, 21], and 2.5 μg/ml cycloheximide to reversibly inhibit protein synthesis [16]. Calcification of cultures treated with glycerophosphate was verified visually, histologically, or by atomic absorption measurement of calcium content as described previously [7]. Cultures were also treated with growth factor supplements as positive controls for the stimulation of MMP production as specified. Supplements consisted of either 15 μg/ml endothelial cell growth supplement [20] with 60 μg/ml heparin or human recombinant b-FGF (10 to 100 ng/ml) with or without heparin (60 μg/ml).

RESULTS

Calcification of cultures in the present study was carefully controlled. The dose of CaBGP (4 m*M*) was found to produce good matrix calcification while leaving most of the adjacent cells undamaged. The histological appearance of such cultures is shown in Fig. 1. A layer of flattened cells was found at the medium–pellet interface of these cultures and to a lesser extent on the basal culture surface. Cells in the center and deeper parts of the pellet were large and round, resembling hypertrophic chondrocytes. Deposition of mineral was concentrated in this central and deeper zone. Cultures produced large amounts of cartilaginous matrix, similar, although less well orientated, to that seen *in vivo* in the growth plate (Fig. 2a). Figures 2b and 2c show the appearance of hypertrophic chondrocytes and their matrix after calcification.

Calcification of CaBGP-treated cultures was confirmed by staining with alizarin red, determination of total calcium by atomic absorption spectrophotometry, and by X-ray probe analysis in the electron microscope. Calcification of micromass cultures (treated with 4 m*M* CaBGP for 48 h) was inhibited 2.5-fold with 2 m*M* levamisole (from 189 ± 9 down to 74.5 ± 6 μg Ca^{2+} per micromass dot; $n = 4$).

Although cell debris colocalized in some cases with sites of calcification, there was no evidence that the proximity of such calcific aggregates was toxic or caused cell death or degeneration (Fig. 2c). On the contrary, normal, viable cells were frequently seen adjacent to calcific deposits. Furthermore, no matter how close cells were to mineral deposits, there was no evidence of phagocytosis of crystals or accumulation of crystals within cells (examined in unstained sections).

The large, hypertrophic cells commonly pushed cell processes out into the surrounding matrix. Such processes appeared to be involved in pericellular matrix remodeling (Fig. 2c). These cells were comparable with hypertrophic chondrocytes of the intact rabbit growth plate mineralization zone. Examples of condensed, terminal hypertrophic chondrocytes, described by Farnum and Wilsman [26], could not unequivocally be identified in cultures.

Prior to calcification of the micromass cultures, there was little detectable collagenase in the medium capable

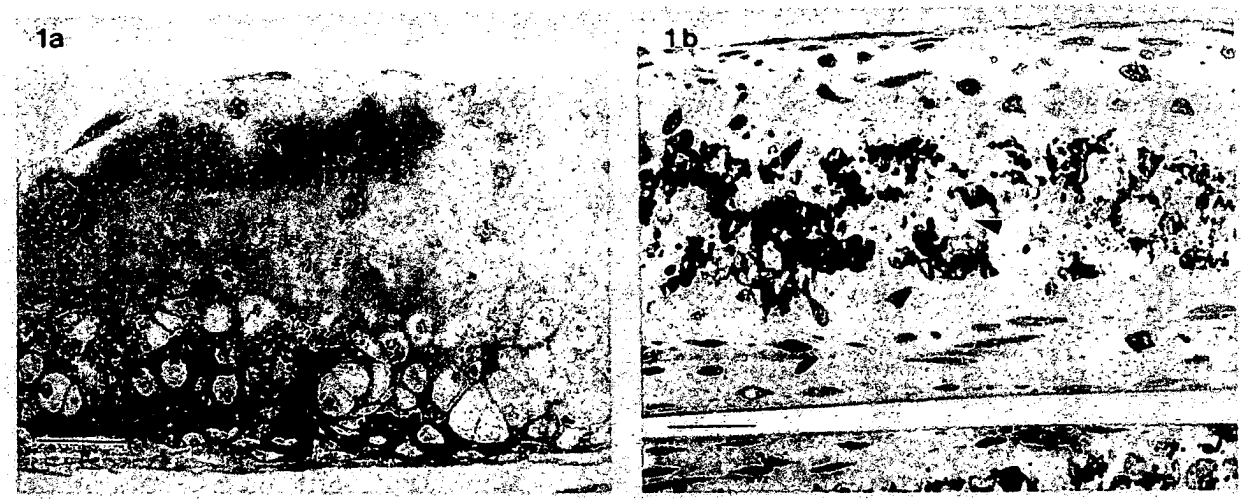

FIG. 1. (a) Structure of a rabbit growth plate chondrocyte micromass culture prior to treatment with CaBGP, i.e., nonmineralized. Flattened cells tended to accumulate at the surface of the culture adjacent to the medium (top of micrograph) and at the base of the cultures. Large rounded "hypertrophic" cells lay mainly in the mid- to deep layers. Traces of calcification were always present due to the slow, spontaneous mineralization which occurred even in the absence of CaBGP. (b) Parallel culture to (a) but after treatment with 4 m*M* CaBGP. Calcification of the central area of the culture is obvious, although large and apparently unaltered chondrocytes (arrowhead) can clearly be seen between the mineral aggregates. Embedded in Spurr's resin and stained with Humphrey's trichrome (bar = 50 μM).

of degrading native types I and II collagen (Figs. 3a and 3b). After treatment with CaBGP, total collagenase activity in the media increased dramatically (approx 17-fold for type II collagen degradation). However, this additional enzyme was almost entirely in the latent form, requiring activation by APMA. The pattern of collagenase production was similar whether assays were performed with types I or II collagen substrate.

Collagenase production was not detectable at 24 h after CaBGP addition but full stimulation was evident by 48 h (Fig. 4a). Although production then declined, it remained elevated for at least 15 days postcalcification. Continuous presence of CaBGP past the 24-h stage was essential to elicit the full increase in collagenase production. Removal of CaBGP after only 24 h (Fig. 4a, last column) reduced the subsequent stimulation of production, measured at 48 h, by almost 14-fold. Addition of levamisole also inhibited collagenase production. Calcification-dependent collagenase production was essentially the same in both the micromass and large-scale cultures.

Figure 5 illustrates the influence of culture age (between 2 and 16 days after plating out) on the degree of stimulation produced by calcification. For both type I and II collagen-degrading enzyme the earliest cultures produced the greatest amount of collagenase. Stimulated production of type II collagen-degrading enzyme fell to approx half in cultures older than 2 days. A similar decrease in stimulated production was evident for type I collagen-degrading enzyme, although in this case after 9 days of culture.

It was also possible to stimulate chondrocyte collagenase production in this culture system, without calcification, by addition of a pituitary-derived growth factor extract (ECGS; 15 μg/ml). Preliminary observations suggest that ECGS- and CaBGP-induced calcification will stimulate approximately the same level of collagenase production but their effects were not additive. In agreement with previous reports [27, 28] pure b-FGF, between 10 and 100 ng/ml (with or without addition of heparin), failed to stimulate collagenase production.

The pattern of gelatinase production was similar, although not identical, to that of collagenase. Figures 3c and 4b show the response of gelatinase production following calcification. Collagenase and gelatinase were assayed in the same conditioned media (see also Figs. 3a and 4a). Unlike collagenase, substantial amounts of gelatinase were produced prior to calcification and half was in the active form. This higher basal activity meant that stimulation of total gelatinase release was more modest than collagenase (1.4- and 1.7-fold increases over premineralized levels in Figs. 3c and 4b, respectively). However, the lag of 24 to 48 h before stimulation and the requirement for CaBGP treatment for 48 h (Fig. 4b), together with inhibition by levamisole, were all features similar to those of the collagenase response.

Cycloheximide was added to cultures, prior to and following calcification, to test whether the increase in collagenase activity represented newly synthesised enzyme or activation of existing enzyme (Fig. 6). Cycloheximide added together with 4 m*M* CaBGP produced a 5.7-fold inhibition of calcification-dependent collagenase pro-

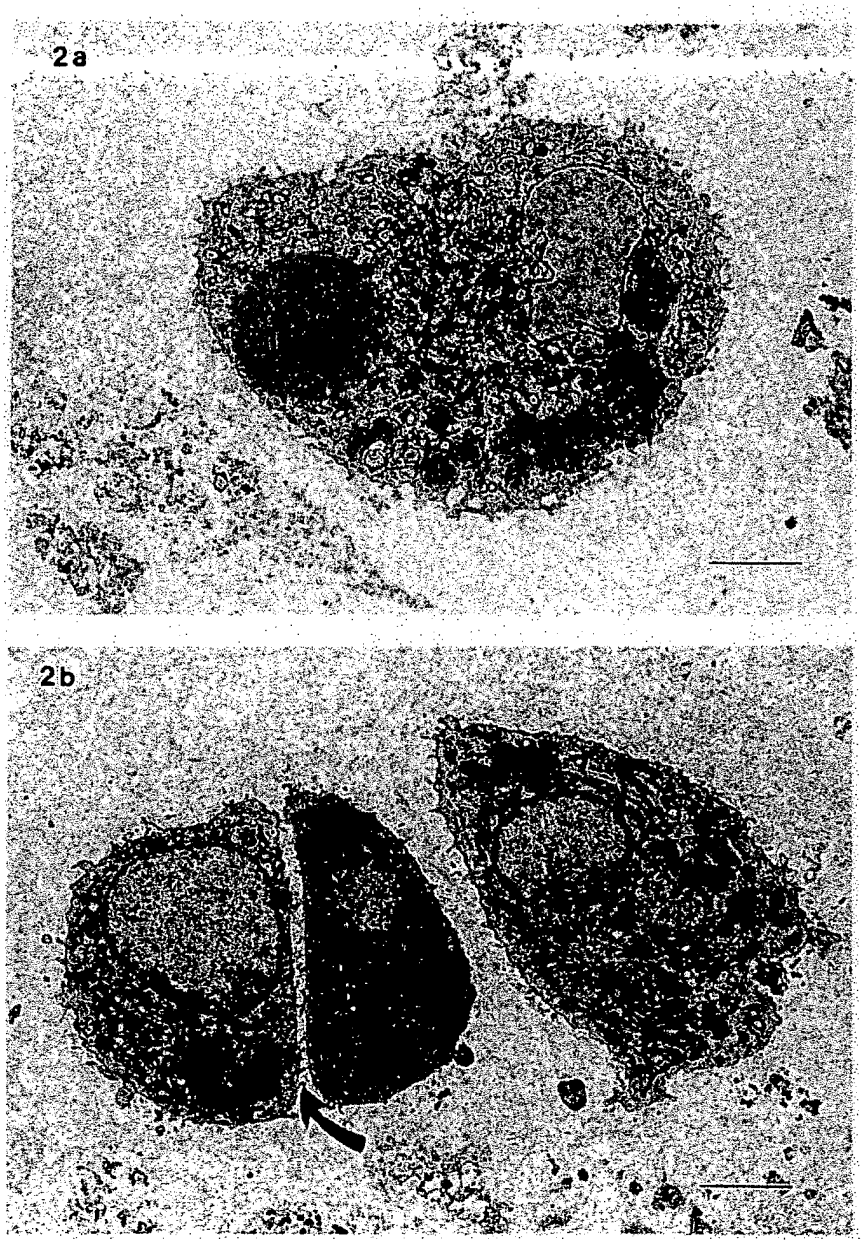

FIG. 2. (a) Electron micrograph of a large chondrocyte in the central layer of a nonmineralized culture, as in Fig. 1a, before treatment with CaBGP. Note the fine fibrous collagenous matrix surrounding the cell (bar = 3 μM). (b) The large rounded cells from an equivalent position in a micromass culture which had been treated with 4 mM CaBGP have essentially the same appearance as those in (a). Cell processes (arrow) appeared to be active in remodeling the fine pericellular matrix at sites around the cells (bar = 3 μM). (c) Detail of a large chondrocyte from a mineralizing culture [as in (b)] showing an aggregate of mineral adjacent to the cell (CA). At the same time a large cell process (P) is pushing out into the matrix (bar = 1.0 μM). Inset: High magnification of the area labeled (P) showing the many small and convoluted processes (arrowhead) and their intimate interaction with the pericellular collagenous fibrils (bar = 0.5 μM).

duction, relative to the calcified control, without cycloheximide. Inhibition of protein synthesis was reversed by removal of cycloheximide for the following 72 h. This resulted in an 8.4-fold increase in collagenase production, a 48% overshoot, relative to the control calcification, which received no cycloheximide. A similar degree of inhibition was seen at the higher dose of 5 μg/ml cycloheximide. When cycloheximide was added 72 h *after* the CaBGP (i.e., after peak stimulation of collagenase production) no inhibition was seen (Fig. 6b).

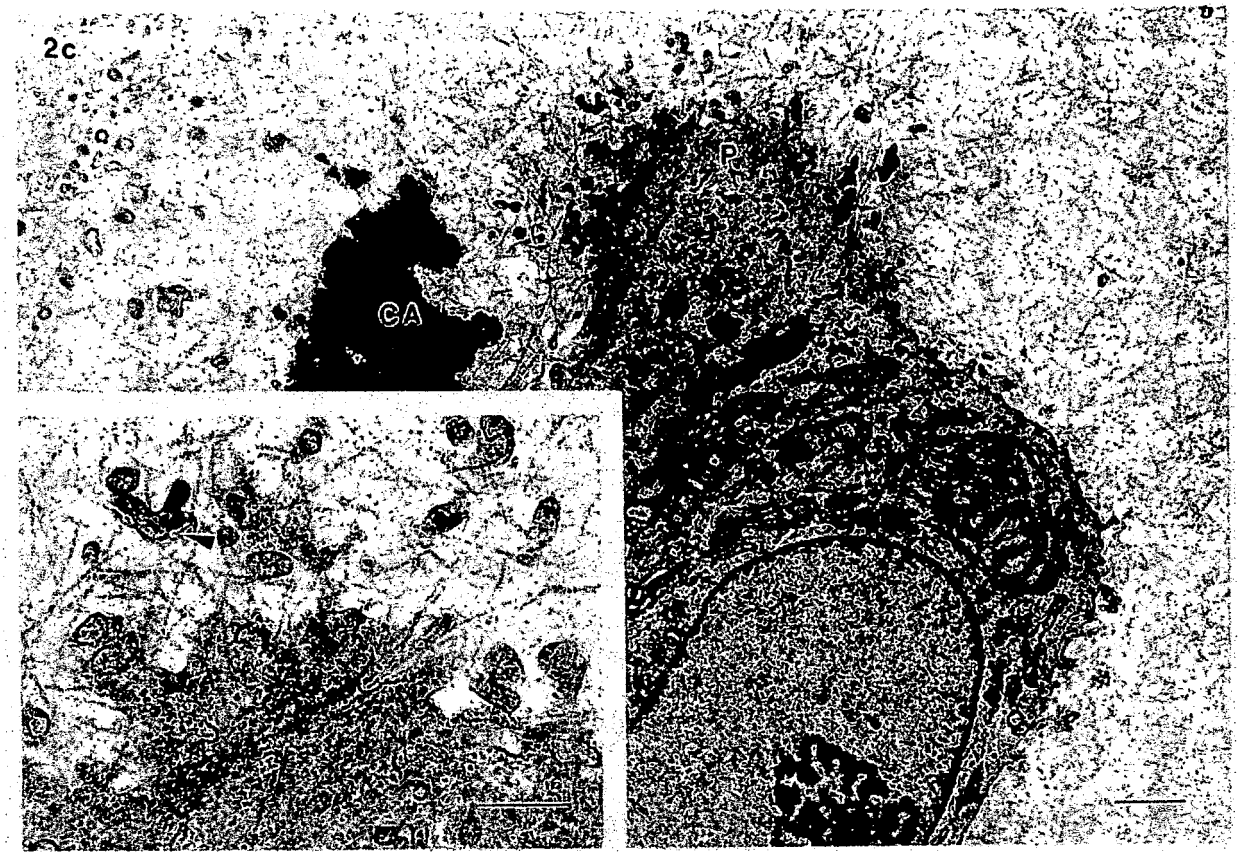

FIG. 2—*Continued*

DISCUSSION

The growth plate chondrocyte culture system, used as a model in this study, has been developed to examine mechanisms of matrix production, calcification, matrix vesicle function, and ESAF production [7, 21]. The present study describes calcification-linked collagenase and gelatinase production by chondrocytes, which may coordinate the matrix lysis associated with both hypertrophy and microvascular resorption. Type II collagen substrate was used here as the most appropriate substrate for chondrocyte derived proteases. Although chondrocyte collagenases will degrade both types I and II collagen, more specific collagenolytic enzymes have been described [22, 29, 30]. The pattern of calcification-linked collagenase release in micromass cultures was no different when measured on type I or type II collagen substrates. However, the delayed age-dependent decrease in type I collagen-degrading enzyme production (relative to type II, Fig. 5) and the lower type II collagen degrading enzyme activity in large-scale cultures was consistent with a collagenase heterogeneity.

Although most forms of adult cartilage resist microvascular invasion [31, 32], a calcification-linked angiogenic invasion of cartilage is pronounced during bone growth. This takes two forms: (i) as cartilage canals [33] and (ii) as metaphyseal capillary sprouts in growth plate [5, 8]. Cartilage canals disappear early in growth [33] but growth plate cartilage angiogenesis at the metaphyseal chondro-osseous interface persists until growth plate closure.

It seems probable that breakdown of cartilage is controlled in the same way whether it occurs at the stage of hypertrophic chondrocyte matrix remodeling or microvascular invasion. Consequently, the control of collagenolysis is likely to be integrated at these two stages, just as cell division, collagen synthesis, and calcification are integrated at earlier stages [2, 34]. It has been proposed that the sudden change in growth plate from an avascular tissue to the site of rapid angiogenesis is due to concerted action of both microvascular cells and hypertrophic chondrocytes [12, 16]. Other authors have stressed the importance of the balance of protease inhibitors [35, 36].

At each scale of the chondrocyte culture system used here, collagenase production was calcification-linked, al-

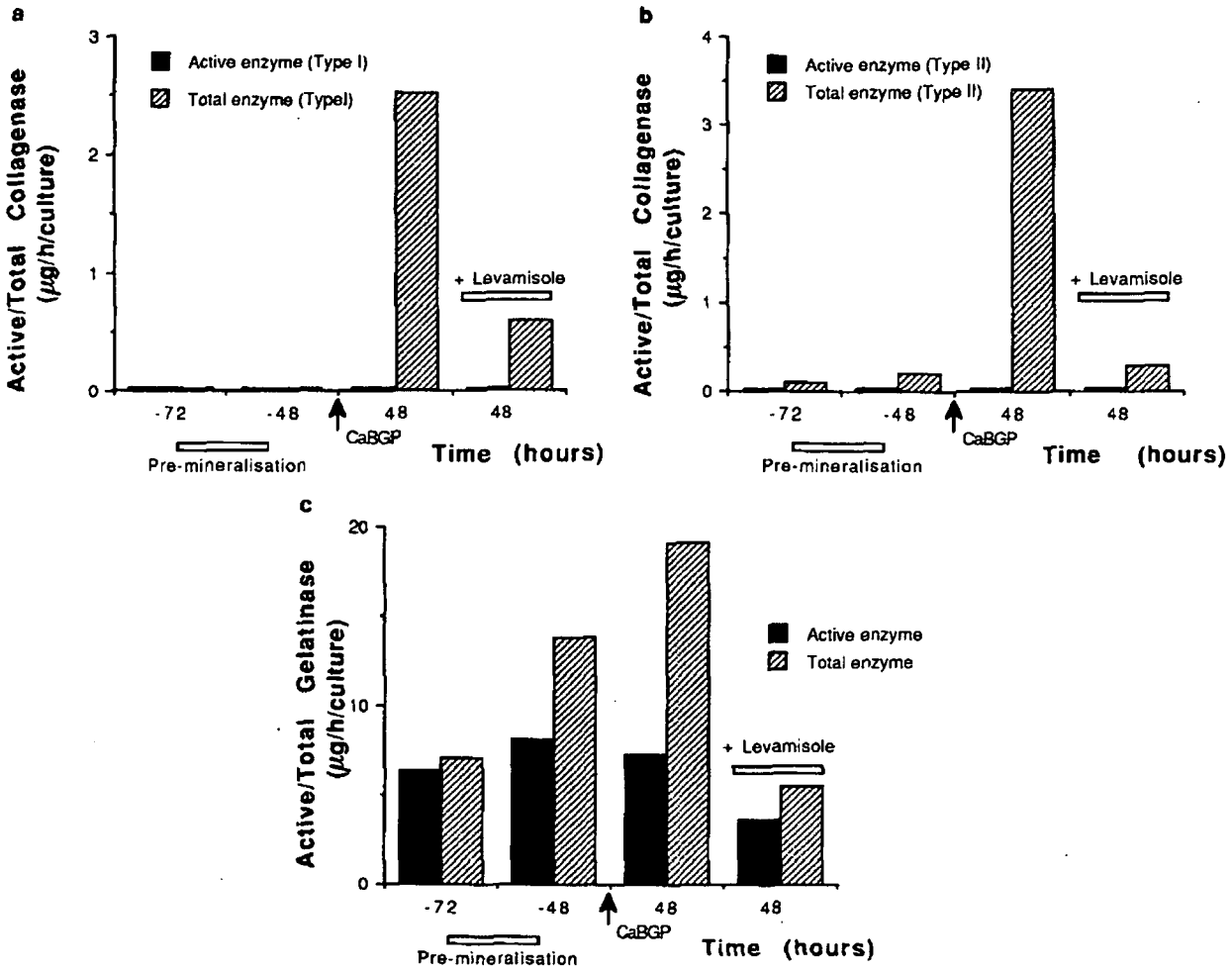

FIG. 3. Micromass cultures of chondrocytes (two dots/culture) were treated with 4 m*M* CaBGP, with or without 2 m*M* levamisole, to produce calcification. After 48 h conditioned media were collected for assay of active and total collagenase using (a) type I, (b) type II collagen, or (c) gelatin substrates. Basal activity was determined in serum-free conditioned media collected 72 and 48 h before CaBGP addition (i.e., premineralization). Total and active enzyme levels in 48 h postcalcification conditioned media were distinguished using APMA activation. The effect of levamisole, also after 48 h, is shown in the last column. Active collagenase was not detectable in any of these cultures.

though a direct, causal dependence remains to be established. This linkage was supported by the minimal enzyme production of noncalcifying (CaBGP-free) cultures and reduced production where calcification was inhibited (+levamisole). Furthermore, it is particularly important that most of the collagenase produced following calcification was in the latent form. This is consistent with the suggestion that only the potential for collagenolysis is increased [12, 16]. Therefore, it is possible that a further activation stage is necessary, although not available, in this culture system.

Inhibition of calcification-linked collagenase release by cycloheximide demonstrates that *de novo* protein synthesis is involved, rather than secondary changes in the APMA-activatable enzyme pool or reactivation of existing enzyme. Increased collagenase activity after removal of cycloheximide (216-h stage), together with the 48% overshoot in recovery in Fig. 6a, suggests that synthesis of a collagenase inhibitor (probably TIMP) had also been blocked.

Both activated endothelial cells [9] and hypertrophic chondrocytes [4, 11, 35] can produce MMPs, including collagenase, while the chondrocytes rapidly remodel their matrix [1, 3] and have been implicated in the production of ESAF [7, 12], which is known to be calcification-dependent [17]. The present study has shown that production of chondrocyte latent collagenase is similarly calcification-linked. Despite the stimulation of both latent protease and activator (ESAF) no detectable increase in actual breakdown was seen following *in*

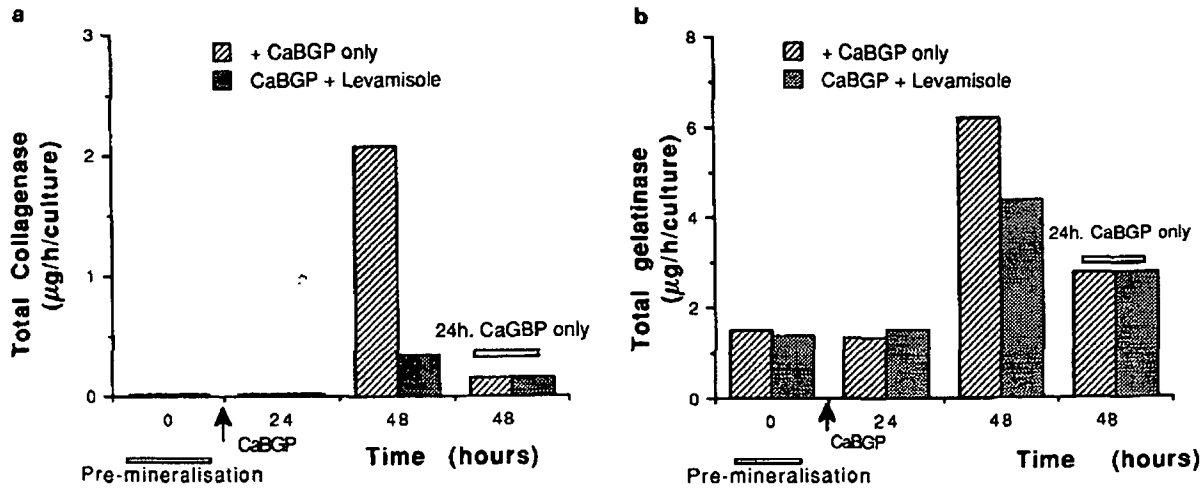

FIG. 4. (a) Levels of total collagenase in pooled micromass conditioned media monitored on type II collagen substrate are shown at 24- and 48-h stages postcalcification. Cultures were given CaBGP throughout both stages. Enzyme release to the media over a 72-h premineralization period is shown in the time 0 column. At each time point the effect of levamisole, added with 4 m*M* CaBGP, is shown. Columns represent enzyme activity in pooled conditioned media from four cultures each, expressed on a per culture basis. The last column shows release of enzyme at the 24- to 48-h stage when CaBGP had been removed after only 24 h. (b) Levels of gelatinase activity released from the same pooled quadruplicate cultures as those in (a). Again, 2 m*M* levamisole (plus CaBGP) was added to parallel culture and the last column shows the short (24 h) CaBGP treatment.

vitro calcification [16]. The ultrastructural studies described here support the observation that there is little extra matrix breakdown following calcification [16]. It has been proposed that calcification-linked ESAF production alone is insufficient to counteract the inhibitory levels of TIMP and that an additional, external stimulus is present *in vivo* [12]. Endothelial cell-derived plasminogen activator [37] or fibroblast growth factor [38], which may promote collagenolysis *in vivo,* are clearly not available in this system. Alternatively, it may be that ESAF has a greater affinity for gelatinase than for collagenase. The increased collagenase production seen in this study when chondrocytes were treated with mixed growth factors, including FGF (but not with pure b-FGF), is consistent with previous reports [27, 28].

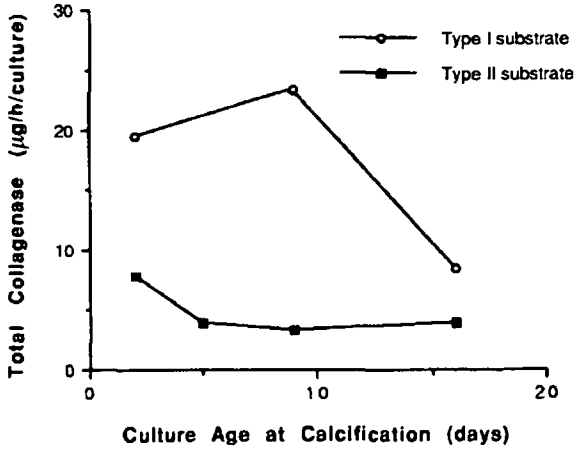

FIG. 5. Plot of the age-related decrease in collagenase stimulation following calcification. Large-scale cultures (initial inoculum 2 × 10^6 cells) were maintained for periods of 2, 5, 9, and 16 days prior to addition of 4 m*M* CaBGP. Conditioned (72 h) serum-free CaBGP media were collected and assayed for collagenase on types I and II collagen.

The ultrastructural study highlighted important similarities and differences between this culture system and growth plate cartilage *in vivo.* Flattened cells were at the surface and hypertrophic chondrocytes segregated to the deeper areas, where mineral accumulated, but these cells did not lie in columns (i.e., normal tissue polarity was lost). There was no indication of a local toxic response to crystals which might result in increased enzyme production. Cheung *et al.* [39] reported increased collagenase activity following phagocytosis of calcific aggregates by articular chondrocytes in monolayer culture. However, no evidence for crystal uptake was found here following calcification. Consequently, the incidence, if any, of crystal phagocytosis was too low to represent a reasonable mechanism for stimulation of cells in this case. Articular and growth plate chondrocytes may behave differently or, conversely, crystal phagocytosis may be a feature of monolayer cultures.

Production of gelatinase was also calcification-linked, although to a lesser degree than collagenase. The present observations agree both with reports of gelatinase synthesis by chondrocytes [28] and its localization in growth plate cartilage [10]. In the growth plate, gelatinase was localized mostly in the upper zones, away

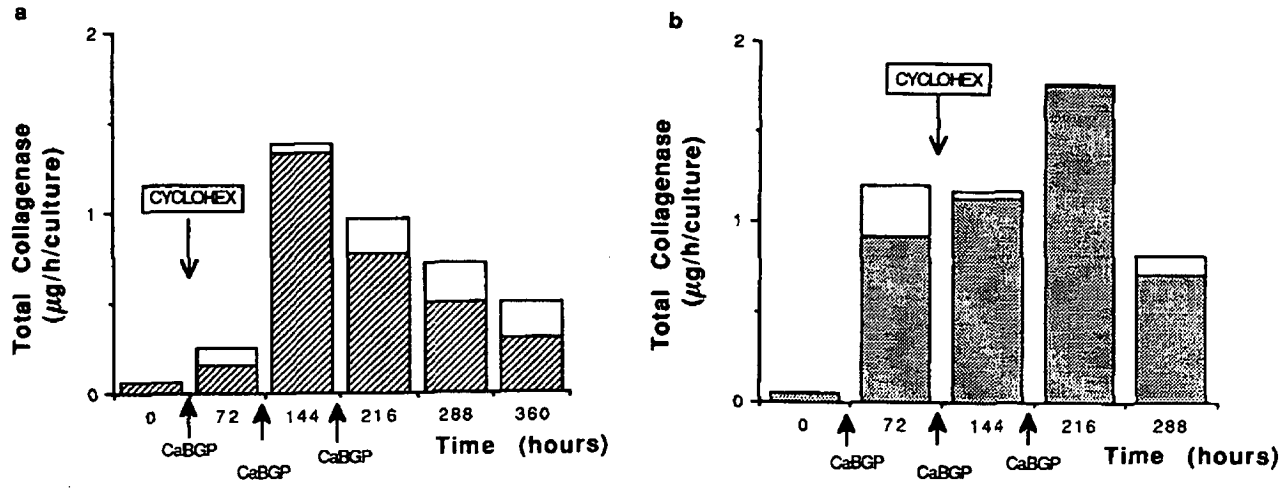

FIG. 6. (a) Effect of inhibition of protein synthesis (by addition of 2.5 μg/ml cycloheximide, CYCLOHEX) on calcification-dependent collagenase production. Basal, premineralization release of collagenase was monitored over 72 h. CaBGP (4 m*M*) was added to the micromass cultures, as before, at 0, 72, and 144 h (arrows). Cycloheximide was added only at the Time 0 point. Protein synthesis was therefore inhibited at the start of mineralization and allowed to recover after 72 h. Mean total enzyme release (n = 3 cultures) was assayed on type II collagen substrate. Unshaded columns represent ±1 SEM. (b) In an experiment parallel to (a) the cycloheximide was added, not at Time 0, but 72 h after initiation of calcification with 4 m*M* CaBGP. Once again protein synthesis in the cultures was allowed to recover by removal of the cycloheximide after 144 h. Mean total enzyme release (n = 3 cultures) was assayed on type II collagen substrate. Unshaded columns represent ±1 SEM.

from sites of mineralization. It is of interest that so much gelatinase was active in cultures where the collagenase remained latent, suggesting that it was not fully inhibited by TIMP. In view of this pattern of activity the role of gelatinase in growth plate may merit reexamination.

The existence of these calcification-linked chondrocyte responses suggests that chondrocytes may monitor changes in extracellular matrix calcium. Recent work on parathyroid cells [40], osteoclasts [41], and keratinocytes [42] suggests that there is a cell surface calcium receptor capable of stimulating intracellular second messenger events. Embryonic chondrogenesis can be regulated by extracellular calcium levels [43], apparently through release of intracellular calcium [44]. Therefore, it is possible to speculate that the calcification-linked cell responses described here are mediated by a divalent cation (e.g., calcium) receptor(s) on the surface of chondrocytes.

The pathophysiological implications of this calcification-linked response are considerable where degenerative matrix changes occur at sites of abnormal calcification. One example would be in osteoarthritis and associated osteophytosis, where both cartilage calcification and increased levels of MMP are found [6, 45].

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